Sterol Regulatory Element-Binding Protein 1 (SREBP-1) Inhibitors Arrest Tumour Growth in Glioblastoma Multiforme (T98G) and Human pancreatic carcinoma (MIA PaCa-2) Cell Lines.

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ABSTRACT

Glioblastoma multiforme (GBM) is the most aggressive and prevalent type of primary malignant brain tumour of the central nervous system, predominantly affecting adults. The current medical treatment for GBM is surgical resection combined with ionizing radiation (IR) and/or chemotherapeutic with temozolomide. However, this treatment is proved to be insufficient as it only provides GBM patients with a one year survival period post-diagnosis. GBM cells have been found to develop resistance to temozolomide treatment. Therefore, the discovery of novel therapeutic agents that possess the capability to pass through the blood brain barrier is warranted in the effective management of GBM.

Sterol regulatory element-binding protein 1 (SREBP-1) is a key transcription factor involved in the regulation of lipogenesis through regulating gene expression of proteins involved in fatty acid biosynthesis. In GBM and many other cancer types, overexpression of SREBP-1 has been found to be associated with its aggressive pathological features. Therefore, SREBPs can be potential targets for therapeutic treatment in GBM. We hypothesize that Indip and other SREBP1 inhibitors such as PF429242 and fatostatin arrest the proliferation of cells in a well-established model of GBM cells, the T98G cell line, maintained *in vitro* in culture.

The present study investigates the efficacy of SREBP-1 inhibitors such as fatostatin, PF429242 and a recently designed short cell penetrating bioactive TAT-fused peptide, indip, in inhibiting the activation of SREBP-1 protein as well as proliferation of T98G cells. All three compounds, fatostatin, PF 429242 and indip led to significant reduction in T98G cell growth in a concentration and time-dependent manner. Our data confirmed that this effect is due to inhibition of SREBP-1

activation that led to T98G cell line apoptosis resulting in reduction in cell size, cell migration and tumor sphere growth. The apoptotic death induced in T98G cells was supported by a more than two fold increase in caspase-3 activity. The three drugs employed arrested the proliferation of T98G cell line in the G2/M phase of the cell cycle. The inhibition of SREBP-1 resulted in significant suppression of expression of key downstream lipogenesis enzymes, Stearoyl-CoA desaturase-1, fatty acid synthase and 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase in both GBM (T98G) and human pancreatic carcinoma (MIA PaCa-2) cells. Furthermore, the combination of Indip (indirect) and PF429242 (direct) treatment had the greatest effect on promoting apoptosis (87% cell death), compared to single drug treatment (40 % by Indip alone and 60% by PF429242). These data suggest that combination treatment with SREBP-1 inhibitor(s) along with temozolomide could be considered as a novel therapeutic approach in the effective management of GBM.

LIST OF ABBREVIATIONS

Glioblastoma multiforme (GBM)

Sterol regulatory element-binding protein 1 (SREBP-1)

fatty acid synthase (FASN)

insulin-like growth factor 1 (IGF-1)

epidermal growth factor receptor (EGFR)

mammalian target of rapamycin complex 1 (mTORC1)

mammalian target of rapamycin complex 2 (mTORC2)

hexokinase 2 (HK2)

glucose transporter 1 (GLUT1)

acetyl-CoA carboxylase (ACC)

Glycogen synthase kinase-3 beta (GSK-3β)

3-hydroxy-3-methylglutaryl-CoA lyase (HMG-CoA lyase)

3-hydroxy-3-methylglutaryl-CoA reductase (HMGCoAR)

tricarboxylic acid cycle (TCA cycle)

Phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)

Protein kinase B (PKB) (Akt)

Guanosine triphosphatases (GTPases)

endoplasmic reticulum (ER)

Sterol regulatory element-binding protein cleavage-activating protein (SCAP)

insulin-induced gene (Insig)

site 1 proteases (S1P)

site 2 proteases (S2P)

the sterol regulate element (SRE)

methyltransferase (MGMT)

Temozolomide (TMZ)

Chemokine ligand 2 (CCL2)

C-C chemokine receptor type 2 (CCR2)

Hypoxia-inducible factor-1 (HIF-1)

high murine double minute 2 (Mdm2)

Tumor protein p53 (P53)

Phosphatase and tensin homolog (PTEN)

Vascular endothelial growth factor (VEGF)

stearoyl-CoA desaturase-1 (SCD-1)

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Chapter 1

GENERAL INTRODUCTION

1.1 Lipid metabolic reprogramming and cancer pathogenesis

1.1.1 The similarities between embryonic and cancer cells metabolism

Over fifty years ago, it was noted that neoplastic tissues within the body are able to synthesize lipids [1] in a way that is very similar to the function of embryonic tissues. Later, in 1996, research by Kuhajda found that a prognostic marker in breast cancer called OA-519 corresponds to fatty acid synthase (FASN)[2]. Since then, more research has determined that tumor cells reactivate de novo lipogenesis as well [3]. Some cancers, including breast and prostate [4–6], have demonstrated increased instances of FASN, which also suggests that fatty-acid synthesis plays a vital role in metabolic reprogramming and cancer pathogenesisin [3].

1.1.2 The main characteristics of malignant tumors

Over the past few years, the field of cancer metabolism has undergone something of a resurrection via new information and technologies. Recent studies have indicated that tumor cells actually reprogram their metabolic networks—a process that attempts to meet the demand for extreme growth and separation that is typically associated with these cells [13–15]. The main characteristics of malignant tumors are twofold: first, the Warburg Effect, which exhibits enhanced glycolysis while a tumor maintains normoxic conditions, and second, an increased glutamine metabolism within the tumor itself [16–19]. Additionally, exacerbated lipogenesis is also a recent hallmark of cancerous tumors [20]—a characteristic that research has proven correlates positively and directly with enhanced glucose and glutamine metabolism

[21–23]. Considering these concepts in juxtaposition with each other, evidence appears to suggest that metabolism reprogramming could be a major player in determining and controlling malignant tumor growth [17]. This has produced a promising strategy for treating cancer: using targeted altered cellular metabolism during de novo lipogenesis. This strategy could be effective because malignant gliomas contain lipid levels in the tumor tissue that are higher than normal tissue, indicating a correlation that could respond positively to this method [18]. Accelerated cholesterol and lipogenesis are hallmarks of cancer and are key elements in the process of malignant transformation. The construction of new cell membranes and maintenance of active signaling in cancer cells relies upon this high intensity of de novo lipogenesis and uptake in lipid rafts.

1.1.3 Insulin resistance and Cancer

Certain alterations in the metabolism of lipids can increase predisposition to and likelihood of cancer development in obese patients. In terms of risk association, obesity is a leading factor associated with several types of cancer in disease risk analysis, with current estimates projecting that 20% of all cancers and 50% of esophageal and endometrial cancers can in fact be attributed to obesity [19]. Obesity contributes to high cancer risks might as a result of an acquired insulin resistance. This is caused by the gradual buildup of lipids in liver and muscle cells, which leads to a high availability of intracellular ceramide and diacylglycerol, inhibiting insulin-stimulated glucose transport and impairing insulin signaling[20]. In turn, this process leads to enhanced secretion of insulin from pancreatic β-cells, increasing the level of insulin-like growth factor 1 (IGF-1) by markedly reducing IGF-binding proteins 1 and 2 [21]. Research shows

that both insulin and IGF1 are protumorigenic growth factors that can stimulate cancer cell proliferation[22]. Increased insulin resistance can also be caused by chronic inflammation [23]. Research using mouse models of genetically- or diet-induced obesity have revealed that the generation of hepatocellular carcinoma is noticeably reliant on the production of inflammatory cytokines as well [24].

1.2 Lipogenesis Pathway

1.2.1 Epidermal growth factor receptor (EGFR) regulates SREBP-1 activation

Several Studies have shown that mutated and / or amplified epidermal growth factor receptor (EGFR) have been shown to occur in 50% of GBM patients [25]. Recent study has shown oncogenic EGFR signaling promotes SREBP-1 activation through inhibiting SCAP by enhancing miR-29 expression, which in turn upregulates its downstream genes to support rapid GBM growth [26]. In addition to directly activating SREBP-1, EGFR signaling in human glioblastoma multiforme activates SREBP1 in a mammalian target of rapamycin complex 1 (mTORC1) independent manner [27]. These findings demonstrate that EGFR signaling activates SREBP-1 through elaborate feedback mechanisms.

1.2.2 PI3K/Akt signaling and SREBP-1

PI3K/Akt signaling contributes to enhanced glucose uptake and glycolysis [28] by regulating glucose transporter 1 (GLUT1) and hexokinase 2 (HK2) [17-18]. Glucose is required to maintain high ATP-levels and also to support the synthesis of NADPH and macromolecules, such as lipids and nucleic and amino acids [18]. PI3K/Akt signaling also works to promote fatty acid

synthesis[30-31]. In turn, the fatty acid synthesis pathway is highly regulated by SREBP-1, which then controls the expression of acetyl-CoA carboxylase (ACC) and FASN [32]. FASN, which mediates the final step of the synthesis of palmitate, is known to be upregulated via PI3K/Akt signaling [30-31]. Additive glycolysis is a key identifying trait in cancers, even when these cancers exist in oxygen-rich conditions. Cancer cells conglomerate glucose to synthesize fatty acids in order to promote the formation of new cell membranes and lipid raft [30]. Taken together, PI3K/Akt signaling plays an important role in activating and promoting glycolysis in which SREBP-1 is a major component in the process of integrating the Glucose carbon flux from PI3K/Akt signaling, as regulated by aerobic glycolysis to fatty acid synthesis.

1.2.3 SREBP-1 integrates glucose and glutamine metabolic influx to de novo lipogenesis

The metabolic flux from glucose and glutamine is regulated by PI3K/Akt signaling and C-Myc [33]. SREBP-1 has been proven to play an important role in interconnecting glutamine metabolism and oncogenic signaling-regulated glucose metabolism to de novo lipogenesis [34]

First, several studies have shown that SREBP-1 activation is closely regulated by PI3K/Akt signaling pathway in cancer cells, in addition to being regulated by sterols [35]. Two possible mechanisms exist in explanation of this kind of regulation. The first mechanism implies that active Akt can enhance stabilization of SREBP-1's nuclear form and in turn enhance its target genes expression via down-regulating Fwb-7, which is a ubiquitin enzyme that mediates SREBP-1's N-terminus degradation through the inhibition of its regulator, Glycogen synthase kinase-3 beta (GSK-3β)[36]. In the second mechanism, PI3K/Akt signaling pathway can regulate SREBP-1's transcriptional activity and its nuclear localization through mammalian target of rapamycin

complex 1 (mTORC1) [37]. Ricoult SJ's study has shown that oncogenic mTORC1-SREBP signaling plays a major role in supporting aberrant proliferation of cancer cells by inducing de novo lipogenesis process (38).

It has recently become known that glutamine also contributes to lipid synthesis and energy production through the tricarboxylic acid (TCA) cycle [39-40]. Moreover, the oncogene Myc promotes glutamate production by stimulating the glutamine transporter and glutaminase. The elevated glutamate in turn is transported into the mitochondria for α-ketoglutarate synthesis. Afterwards, α-ketoglutarate can then contribute to ATP synthesis by entering the TCA cycle [39-40], or convert into citrate via reductive carboxylation under defective mitochondria or hypoxia conditions to promote SREBP-1 driven de novo lipogenesis [33, 41]. Researchers have recently reported that glutamine can be preferentially chosen by cancer cells as a precursor to the synthesis of fatty acid during an impaired mitochondria condition or the process of hypoxia [33, 41]. A key regulator for lipid synthesis through glutamine metabolism could potentially be SREBP-1. SREBP-1 plays an integral role in connecting oncogenic Myc regulated glutamine metabolic flux and PI3K/Akt signaling-regulated glucose metabolic flux to de novo lipogenesis [42]. Taken together, the development of specific inhibitors made to target SREBP-1 could create a new therapeutic strategy in treating cancer, particularly in cancers with oncogenic Myc and PI3K/Akt signaling.

1.3 Cholesterol

1.3.1 The mevalonate pathway

Lipid metabolism also features another vital biosynthetic process called the mevalonate pathway, which induces the synthesis of cholesterol. Cholesterol biosynthesis involves the formation of 3-hydroxy-3-methylglutaryl-CoA lyase (HMG-CoA lyase) by combining acetoacetyl-CoA and acetyl-CoA. HMG-CoA is reduced to mevalonate by 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCoAR), which is indicative of the rate-limiting reaction of the cholesterol synthesis pathway and is meticulously regulated. The stability of HMGCoAR, an endoplasmic reticulum-transmembrane protein, is regulated by a sterol-sensing-domain; this mediates its degradation under saturating sterol levels [43]. In addition to forming the structural backbone of the steroid hormones, cholesterol is a crucial component of cell membranes; it controls the fluidity of the lipid bilayer, and it forms lipid rafts, detergent-resistant microdomains.[44]. Furthermore, cholesterol biosynthesis contributes to the geranylgeranylation of Rho and the farnesylation of Ras, which are both required for the isoprenylation of Guanosine triphosphatases (GTPases) [45].

1.3.2 SREBPs are activated in response to low sterol conditions

In addition to being able to escort SREBPs from the endoplasmic reticulum (ER) to the Golgi apparatus, sterol regulatory element-binding protein cleavage-activating protein (SCAP) is also considered as the cholesterol sensor. When sterol levels decrease, SCAP transports SREBPs from the ER to the Golgi apparatus, where SREBPs will be proteolytically activated and help integrate and regulate the process of the cholesterol synthesis. SREBP in turn regulates the expression of cholesterol synthesis enzymes (46).

1.3.3 HMGCoAR is the target for statins

Cholesterol buildup has been demonstrated in prostate cancer (47), while alteration of the mevalonate pathway has been closely linked with transformation [48]. HMGCoAR is the target for statins, which are a class of cholesterol-lowering drugs. Several cancer-cell lines demonstrate antiproliferative activity through statins, with cell cycle arrest in breast cancer cells [49] and apoptosis in acute myeloid leukemia [50]. Furthermore, statin treatment has been shown to enhance the sensitivity of chemotherapeutic agents toward colorectal cancer cells by inducing epigenetic reprogramming, and a complex combination of statins with chemotherapy has demonstrated encouraging results in patients afflicted with hepatocellular carcinoma [51] and acute myeloid leukemia [52]. Cancer incidence is clearly affected by statins, but the effects' severity and usefulness are problematically dependent on the tumor type and the class of the statins that are integrated into a given procedure.

1.3.4 Cholesterol esters

Several researches have shown that cholesterol esters have only been observed in high-grade gliomas [53]. Cholesterol esters have also been found in human urothelial carcinoma and renal cell carcinomas [54-55], but are absent in the corresponding healthy tissues of these cells. By compiling this information together contextually, research appears to demonstrate that cholesterol esters can be a future biomarker in diagnosing the malignancy of specific tumors [56]. Given that free cholesterol levels are strictly controlled through negative feedback mechanisms, formation and accumulation of nascent cholesterol esters could be the method glioma cells use to store cholesterol. Cholesterol esters might quickly release cholesterol for both growth and survival mechanisms in cancer cells, particularly when cells require cholesterol

in the first place. Therefore, as cholesterol esters do not exist in healthy brain tissue, stopping cholesterol ester formation and accumulation may be a promising therapeutic strategy in attempting to inhibit cancer growth.

1.4 SREBPs

1.4.1 SREBPs activation pathway

These SBREPs are translated as 125-kDa precursors, which are then included in the endoplasmic reticulum membrane, where they are held by the SREBP cleavage-activating protein (SCAP). SREBP1a, SREBP1c and SREBP2—three SREBP isoforms—have been recognized in the cells of mammals [57]. SREBP1a and SREBP1c are created through alternative splicing and differ in their expression levels across various types of cell tissues; SREBP1a is the most prominent isoform in cultured cell lines [57]. Despite there being an overlap in their target gene functionality, SREBP1 primarily regulates phospholipid, fatty acid and triacylglycerol synthesis, whereas SREBP2 monitors and dictates the expression of cholesterol-synthesis genes [57-58]. In healthy tissues, SREBPs activation is closely regulated by intracellular sterol levels via negative feedback mechanism[59].

The N-terminal domain is a basic-helix-loop-helix lucine zipper and functions as the transcription factor. These proteins are synthesized as ER membrane proteins and release from the ER is required for activation. SREBP is found as a complex with SCAP and insulin-induced gene (Insig) in the ER [57]. Binding of Insig to SREBP-SCAP blocks translocation of the SREBP-SCAP complex to the golgi apparatus, where SREBP becomes activated. Sterol deprivation promotes the dissociation of Insig from the SREBP-SCAP complex promoting movement to the

golgi apparatus [57]. In the golgi SREBP-1 can be cleaved by site 1 and site 2 proteases [58]. Thus, the N-terminal of SREBP-1 translocates to the nucleus and binds the sterol regulatory element (SRE) promoting expression of lipogenic genes [58]. . SREBP-1 has been found to regulate the expression of a number of proteins involved in fatty acid synthesis including; acetyl-CoA carboxylase, acetyl-CoA synthetase, acetyl-CoA binding protein, ATP citrate lyase, fatty acid synthase, peroxisome proliferator-activated receptor-γ, malate dehydrogenase and stearoyl-CoA desaturase [59]. SREBP-1 plays an integral role in connecting PI3K/AKT signaling regulated glucose metabolism and Myc-regulated glutamine metabolism to lipogensis [57]. In addition to regulating several cellular processes, such as membrane biogenesis, autophagy phagocytosis, immunity and hypoxia and cell cycle [57]. In healthy tissues, SREBPs activation are closely regulated by intracellular sterol levels via negative feedback mechanism[59].

1.4.2 SREBP-1 and mTORCs

SREBP-1 is highly expressed in cancers [18-20]. Targeting SREBP-1 through genetic and pharmacological methods has also demonstrated significant inhibition of tumor cell growth, which proves that SREBP-1 is a potentially promising, if novel, molecular target in cancer treatment. SBREP-1 regulation is also known to be much more complex in cancer cells. Studies show that using rapamycin to inhibit mTORC1 has a diminutive effect on SREBP-1 nuclear localization and its transcriptional level, but inhibiting its upstream factors, such as EGFR, PI3K, and Akt, can significantly decrease SREBP-1 activation and its nucleus localization [60]. This data suggests that other oncogenic molecules outside of the already present mTORC1 may play important roles in SREBP-1 regulation, in addition to the above mentioned reason. According to

the results from the mTOR kinase inhibitor Torin-1 [61] (the inhibitor of both mTORC1 and mTORC2 activity), mammalian target of rapamycin complex 2 (mTORC2) may also play important rule in stabilizing the nuclear form of SREBP-1 [62]. These results also show a significant decrease in SREBP-1 distribution in the nucleus, especially in comparison to mTORC1 inhibitor rapamycina lone [63]. Finally, this process could, at least in part, digress and reveal the reason that targeting mTORC1 has had an incrementally promising response in clinics using rapamycin or rapamycin derivatives in cancer treatment [64]. Given that lipogenesis is an inextricable source in the formation of cellular membranes, cancer cells may use redundant cellular pathways to regulate the expression of SREBP-1 and its activation, in order to guarantee that cells will have the ability to obtain enough lipids for their rapid proliferative processes.

1.5. Glioblastoma multiforme (GBM)

1.5.1 The cellular origin for GBM

GBM develops from astrocytes, star-shaped glial cells, that play a major role in supporting nerve cell function. Astrocytes are the most abundant (20-40%) glial cell in the CNS. They perform many functions including regulating electrical impulses, provide glucose and lactate to neurons, facilitate neuronal repair and in regulating blood flow [65]. GBM develops primarily in the cerebral hemispheres with 95% arising in the supratentorial region, though it can also develop in other parts of the brain, brainstem, or spinal cord [66].GBM has rapid and enormous capacity to invade neighbouring brain structures. Histologically GBM is quite heterogeneous featuring cystic and gelatinous areas, necrosis and multifocal hemorrhage [67]. GBM tumors are recognized clinically by a single, relatively large, irregular shaped lesion and an abnormal

vasculature having glomeroid tufts and hyper proliferative, leaky and unorganized blood vessels[68]. The current available standard medical treatment for GBM is surgical resection combined with ionizing radiation (IR) and chemotherapy (temozolomide, carmustine, or lomustine) [66]. This treatment is not sufficient and only provides a very minimal extension of survival (12–14 month) post-diagnosis [68]. Thus, novel therapies are necessary to decrease the morbidity and mortality rate of patents diagnosed with GBM.

1.5.2 Brain Tumour: Statistics

On average, an individual diagnosed with GBM will live between 12 to 15 months, where the mean incidence rate of this condition is 3.19 in 100, 000 individuals within a population. Temozolomide (TMZ) is an alkylating agent and the current drug of choice for treating GBM; however, even with aggressive treatment, involving radiotherapy and chemotherapy, most patients will relapse. Treatment with radiotherapy and surgery has a dismal 3- and 5-year survival rate of 4.4% and 1.9% respectively [69]. The addition of temozolomide (chemotherapy) increased the 3- and 5-year survival rate to 16% and 9.8% respectively [69].

1.5.3 Current state of treatment for GBM

The genomic instability, high proliferation rate, high survivability of GBM tumor cells and the development of glioma cancer stem cells promotes significant resistance to current therapeutic interventions [68] Dysregulation of critical signal transduction pathways is critical in GBM propagation, survival and metastasis. Studies have shown that very high levels of 6-methylguanine DNA methyltransferase (MGMT), overactivity of PI3K/Akt pathway, over expression of EGFR, CCL2/CCR2 axis, hypoxia-inducible factor-1 (HIF-1), high murine double

minute 2 (Mdm2) activity, galactin-1 expression, and mutations in tumor protein p53 (P53) and phosphatase and tensin homolog phosphatase and tensin homolog (PTEN)

oncogenes play important roles in GBM progression[70-71]. Current studies have focused on targeting tumour-specific genes to suppress tumour growth [70-71]. Bevacizumab is a humanized monoclonal antibody that blocks vascular endothelial growth factor (VEGF) and suppresses angiogenesis. Bevacizumab is used in combination therapy for GBM, the effects have been varied or inconclusive, thus concluding bevacizumab benefits and continued use[72-73]. EGFR inhibitors (nimouzumab , cetuximab, dacomitinib) have been also been used in combination therapy for GBM and results were also varied [74-75]. Because GBM is a highly dynamic and a heterogenetic tumor, targeting a specific gene has been very inefficient as a therapeutic approach due to the ability of cancer cells to compensate for disrupted pathways. Thus, targeting a global system may lead to a more feasible option to aid in the design of combinatorial regimens.

1.5.4 GBM and SREBP-1

Recent research on mechanisms of how cancer cells grow rapidly and sustain themselves indicates that in malignant cells, lipid metabolism undergoes reprogramming to meet high metabolic demands of cancer cells and the lipogenic phenotype is a major characteristic of cancer. Tumor cells even divert glucose and glutamate metabolism pathways to serve in lipid biogenesis process. SREBP-1 has been shown to be a major part of many other cellular functions, such as cell size, proliferation, and the general regulation of cell cycle [76]. Indeed, the elevated lipid biogenesis regulated by lipid transcription factor, SREBP-1, is considered a

major characteristic of malignancies including GBM [77-78]. In GBM and many other cancer types, SREBP-1 lipid transcription factor and its downstream gene targets are upregulated/over-activated to provide much needed fatty acids and phospholipids in order to promote growth and survival. A better understanding on how lipid biogenesis and SREBP-1 contribute to tumorigenesis and survival of cancer cells may provide new strategies for cancer treatment.

HYPOTHESES AND RESEARCH OBJECTIVE

1.6 Hypothesis and The aims of the research

1.6.1 Hypothesis

Emerging evidence strongly indicates that SREBP-1 is a potential therapeutic target for GBM. Targeting SREBP-1 activation seems to be a more effective anti-cancer therapeutic approach than blocking its upstream or down-stream targets as mentioned in the introduction. We therefore hypothesize that “Indip” , small molecule “PF429242” and Fatostatin by inhibiting SREBP-1 activation and lipid biogenesis in GBM effectively prevent brain tumor proliferation, growth and decrease survival of glioma cells.

1.6.2 Mechanism of Drug Targets and Drug actions

1. Indip is a short cell penetrating bioactive TAT-fused peptide, which can directly inhibit SREBP-1 activation by stabilizing Insig1 an endoplasmic reticulum (ER) membrane localized protein. The degradation of insulin-induced gene-1 protein (Insig-1) is required for SREBP-1 activation.

2. Fatostatin inhibits SREBP-1activation by binding to SCAP and inhibiting SCAP's ability to transport SREBP-1 to the Golgi apparatus.

3. PF429242 suppresses SREBP-1 activation by inhibiting Site-1 protease, since SREBP-1 is proteolytically cleaved by the Golgi associated Site-1 protease and Site-2 protease in order to release the-terminal transcription factor domain of SREBP-1.

1.6.3 The aims of the research

1. Determine the half maximal inhibitory concentrations (IC50) of "Indip", "PF429242" and " Fatostatin" against T98G glioma cell line and tumor stem cell-like cells harvested from GBM patients.

2. To investigate therapeutic effects of SREBP-1 inhibiters by examining cell viability, proliferation, cell cycle arrest, tumor sphere formation and migration rate when compared to the vehicle treated T98G glioblastoma cells

3. To investigate the effects of SREBP-1 inhibitors on SREBP-1 activation and the expression of its target genes by using western blot.

4. Investigating possible synergistic/additive effects of combination therapy of SREBP-1 inhibitors with TMZ in tumor cell cultures

CONCLUSION AND DISCUSSION

1.7 General conclusion

1.7.1 Targeting SREBP-1 to treat cancer

Targeting metabolic alterations has emerged as a new strategy to treat cancer in the past few years. For example, inhibiting glycolysis by using HK2 or a 2-deoxyglucose (2-DG) inhibitors, both of which have shown results that are highly promising, at least in a pre-clinical model [48, 75, 76]. It has also been proven that lipogenesis appears highly activated in cancers and interconnects with both glutamine and glucose metabolism [17, 77, 78]. With this information taken into consideration, the suppression of fatty acid synthesis could offer a new take on blocking tumour growth. SBREP-1 is highly expressed in glioblastoma tissues and its over activation leads to activation of its target genes [18]. Several studies have shown that Inhibition of ACC and FASN activation has been shown to suppress cancer cell growth. Moreover, Genetic knockdown or pharmacological inactivation of SREBP-1 and ACL have been shown to significantly impair cancer cell growth and lead to cancer cell death in vitro and in vivo [18]. SREBP-1 has emerged as a very promising drug target in this regard. It is important to factor in that, in normal tissue, SREBP-1 and its target genes are usually at low levels of activity and expression, as opposed to the high levels they exhibit in cancer cells. Therefore, the specific targeting of SREBP-1-regulated lipogenesis pathways could have an incredibly bright future as a novel approach to treat cancer and malignant tumor growth.

1.7.2 Conclusion for targeting SREBP-1 to suppress the GBM growth

Sterol regulatory element-binding protein 1 (SREBP-1) is a key regulator of lipid and cholesterol biogenesis and has been linked to the development and progression of glioblastoma multiforme. Inhibition of SREBP-1 overactivation by fatostatin, PF429242 or indip treatment resulted in significant reduction of downstream signaling proteins. In addition, these inhibitors

decreased tumor size and induced cell cycle arrest at the G2/M phase, leading to apoptotic cell death. This study provides evidence that SREBP-1 regulates cyclin dependent kinase 5, stearoyl-CoA desaturase and HMG-CoA reductase in GBM cells. Further, combination treatment enhanced the anti-proliferative effect of T98G tumor cells. Thus, it is evident that SREBP-1 inhibitors as individual or in combination may be the novel therapeutic intervention in the management of GBM. Further, treatment of GBM cells with Indip/PF429242 has an additive effect in promoting apoptosis in T98G tumor cells. Taken together, these findings indicate that combinational drug therapy of Indip/PF429242 has an enhanced therapeutic effect over temozolomide. Please see Chapter two for more details. (The study is during patent approval process).

1.7.3 Conclusion for targeting SREBP-1 to suppress the proliferation of MIA PaCa- 2 cells

Overactivation of SREBP1 and elevated lipid biogenesis are considered the major characteristics in malignancies of prostate cancer, endometrial cancer, and glioblastoma. A recent study also showed that inhibition of upstream factors such as EGFR, PI3K and Akt drastically reduced expression of SREBP-1 in cancer cells (60). Sun Y. *et al* have shown a clear correlation of SREBP1 levels with prognosis in pancreatic cancers (79). However, the effect of inhibition of SREBP1 on pancreatic cancer cells is not well studied. The present study examines the effect of suppression of SREBP1 activation by its inhibitors like fatostatin and PF429242 besides analyzing the impact of inhibitory effects on SREBP1 downstream signaling cascade in MIA PaCa-2 pancreatic cancer cells. Both fatostatin and PF429242 inhibited the growth of MIA PaCa-2 cells in a time and concentration-dependent manner. Further, inhibition of SREBP1

overactivation by fatostatin and PF429242 leads to reduced expression of its downstream targets. Our in vitro data suggest that SREBP1 may contribute to pancreatic tumor growth and its inhibitors could be considered as a potential target in the management of pancreatic cancer cell proliferation. Please see Chapter three for more details. (The study was published on Biochemical and Biophysical Research Communications).

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CHAPTER 2

Pharmacological effects of Sterol regulatory element-binding protein 1 (SREBP-1) inhibitors on the Glioblastoma multiforme brain tumor cells – A Novel Therapeutic Approach

ABSTRACT

Glioblastoma multiforme (GBM) has been linked to aberrant fatty acid metabolism and overexpression of sterol regulatory element-binding protein 1 (SREBP-1), a key lipid transcription factor. In the present study, we investigated the anti-tumor effect of novel SREBP-1 inhibitors on glioblastoma brain tumor cells. Our data showed that the direct (fatostatin and PF429242) or indirect (Insig-1-derived interference peptide [Indip]) SREBP-1 inhibitors significantly reduce cell viability, proliferation and migration rate when compared to the vehicle treated T98G glioblastoma cells. Fatostatin, Indip and PF429242 arrested the cell proliferation at G_2/M phase. They impeded cell migration through CDK-5 inhibition and induced apoptosis by increasing caspase-3 activity. SREBP-1 inhibition with fatostatin, Indip and PF429242 also resulted in significant suppression of downstream lipogenesis enzymes like stearoyl-CoA desaturase-1 (SCD-1), fatty acid synthase and 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCoAR) in T98G tumor cells. In addition, the combination of Indip and PF429242 treatment had the greatest effect on suppressing cell growth (87% cell death), compared to individual treatments (40% and 60%, respectively). Our findings identify the anti-tumor potential of SREBP-1 inhibitors on T98G cells. Further, combination of direct and indirect treatment enhances its effect and may be used as a novel therapeutic approach for the treatment of GBM.

INTRODUCTION

Glioblastoma multiforme (GBM) is the most common and deadliest malignant primary brain tumor of the central nervous system (CNS), referred to as glioma[1, 2]. GBM is considered to be a rare tumor affecting 0.1% of the population with a survival rate of 12-14 months and its incidences increased up to 3% per year[2, 3]. GBM are classified as Grade IV (most serious) astrocytoma according to the World Health Organization (WHO) tumor classification and accounts for more than 60% of all brain tumors in adults[4]. Current drug therapy like temozolomide (TMZ) increased the 3- and 5-year survival rate to 16% and 9.8%, respectively[5]. However, new reports indicate resistance to temozolomide with neurocognitive impairments and novel drugs are urgently needed to enhance patient survival and quality of life [2].

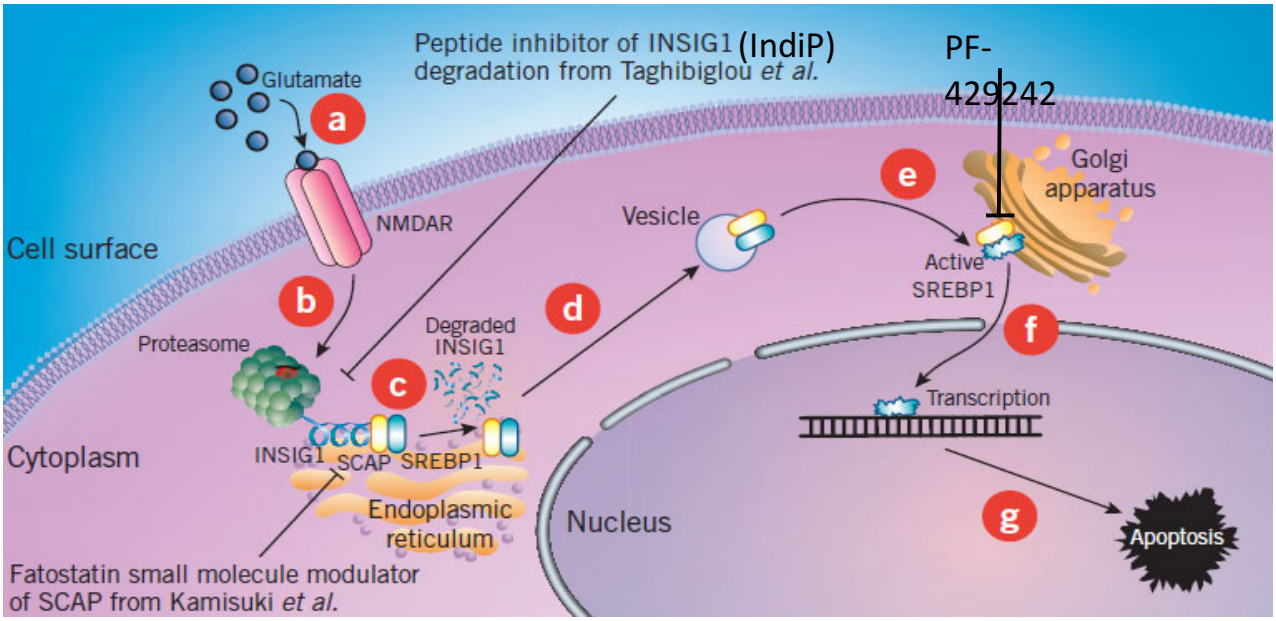
Dysregulation of critical signal transduction pathways has been reported in GBM propagation, survival and metastasis. Malignant cells have been found to increase lipid metabolism to meet the energy needs of highly proliferating tumor cells[6,7]. Tumor cells divert glucose and glutamate metabolism pathways as well to serve in lipid biogenesis process and signal transduction during tumor progression[8,9]. De Novo fatty acid synthesis is the major supply of fatty acids for cancer cells, thus, enzymes involved in fatty acid metabolism have attracted major interest as a therapeutic target for cancer biology. Studies have shown that inhibiting fatty acid synthase or lipogenesis induces cancer cell death[10]. The sterol regulatory element-binding proteins (SREBPs) have gained significant interest as a possible therapeutic target for cancer treatment. SREBPs regulate fatty acid and cholesterol biosynthesis[11]. Elevated lipid biogenesis in GBM is regulated by lipid transcription factor sterol regulatory element-binding protein 1 (SREBP-1)[12]. In many cancers and in particular GBM, SREBP-1 and its downstream gene targets are upregulated promoting lipid biogenesis, tumor cell growth and survival[13]. A better understanding on how lipid

biogenesis and SREBP-1 contribute to tumorigenesis and survival of cancer cells, may provide new strategies for cancer treatment.

Emerging evidence strongly indicates that SREBP-1 is a potential therapeutic target for GBM[14]. Direct inhibition of SREBP-1 activation is a more effective approach than blocking its upstream or down-stream targets for inhibiting lipid biosynthesis and promoting tumor cell apoptosis reducing tumor growth[14]. In addition, high SREBP expression is associated with poor prognosis of GBM[12]. Therefore, inhibition of SREBP-1 could be a potential strategy to target. In this study, the activation and subsequent maturation of the SREBP-1 transcription factor was targeted through drug treatments using Indip. Indip is attached to a trans-activator of transcription (TAT) short peptide consisting of an 11 amino acid sequence. These peptides have shown to be a prospective method of drug delivery at a molecular level as it can cross biologically impermeable membranes, and thus, when fused to potential drug treatments, both can cross the BBB and arrive at their target. Indip, a TAT-fused short peptide, exhibits prospective use in the treatment of GBM as it demonstrates the inhibition of SREBP-1 maturation through the prevention of Insig-1, Insulin-induced gene 1 protein, ubiquitination and then its subsequent degradation, which further inhibits glioma growth and survival**.** We have previously tested Indip in animal models of stroke and ALS and have shown its ability to pass through blood brain barrier (BBB)[15, 16]. We therefore hypothesized that Indip alone or in combination with Fatostatin and/or PF429242 (small molecule SREBP inhibitors) could effectively prevent GBM tumor proliferation, growth and decrease survival of glioma cells *in vitro* and *in vivo*. Our data using the T98G GBM cell line treated with Indip, fatostatin and PF429242 confirms effectiveness of

this therapeutic approach and supports inhibition of SREBP-1 as a potential novel therapeutic for GBM.

Mechanisms of Action of SREBPs inhibitors



(Adapted from Osherovich, L. *SciBX* 2(48); 10.1038/scibx.2009.1750

RESULTS

2.1.1 All three SREBP-1 inhibitors inhibit proliferation in a dose- and time-dependent manner.

Glioblastoma is a very aggressive cancer in part to the high proliferation rate of the cancer cells. To evaluate whether inhibition of sterol regulatory element-binding protein 1 (SREBP-1) activity promotes a decrease in T98G cells (Glioblastoma cell line) proliferation, we carried out a set of assays to determine whether fatostatin (inhibitor of SREBP-1), PF429242 (reversible inhibitor of SREBP-1) and Indip (inhibitor of Insig-1 degradation) inhibits T98G cell proliferation and the mechanism involved.

The T98G cells were incubated (1 x 10^5 cells/well) in tissue culture plates and allowed to attach and spread. The cells were further incubated with media alone or with Indip (30-1,000 μM), fatostatin (2.5-100 μM), TMZ (60-2,000) or PF429242 (2.5-100 μM) to determine the IC_{50} for proliferation (figure 1a-d). The cells were incubated for 24, 48 and 72 h and then analyzed for proliferation using the CCK-8 assay. Fatostatin and PF459245 showed a more potent SREBP-1 inhibition, with an IC_{50} of 5 and 10 μM respectively, compare with Indip and TMZ, with an IC_{50} of 250 and 500μM at 24 h, respectively. The doubling rate for T98G cells is 28 hrs allowing the cells to have gone through one doubling. We found that all the SREBP inhibitors significantly reduced T98G cell proliferation with an optimal concentration range for inhibitor as 2.5-20 μM fatostatin, 10-40 μM PF429242, 125-500 μM Indip and 250-1,000 μM TMZ (figure 1a-d).

Further, we determined the effect of these inhibitors on T98G cell proliferation over time. The GBM cells were plated in tissue culture plates allowed to adhere and spread. The cells were then incubated in 10%-FBS-EMEM alone or with fatostatin (5 μM), PF429242 (10 μM) or Indip (250 μM) for 8 days at 37ºC and 5% CO_2. Each day the cells were analyzed for cell number by manually using a hemocytometer. We showed that both fatostatin and PF429242 significantly prevented proliferation as the cell population hovered around 1 x 10^4 cells, initial number plated, for the duration of the experiment (figure 1e). Both 5 μM fatostatin (FAT) and 10 μM PF429242 (PF) has a 3-6 fold decrease in the number of T98G cell after 8 days compared to untreated (C). However, 250 μM Indip (INDIP) did not significantly prevent proliferation of T98G cells after 4 days compared to untreated group (C). In addition, dansly fluorophore labeled Indip and immunofluorescence staining were used to demonstrate that Indip can cross cell membranes with facilitation of the cell penetrating bioactive TAT as mentioned in the introduction (Figure

1f). Dansly fluorophore labelled Indip was used to track intracellular localization of Indip. The florescent antibody against insulin receptor was used to enhance visual contrast. (Figure 1f). Taken together, all three SREBP-1 inhibitors inhibit proliferation in a dose- and time-dependent manner and treatment with PF429242 had the most significant long term antiproliferative effect in T98G cells.

To verify that fatostatin, PF429242 and Indip inhibit SREBP-1 activation and its downstream genes upregulation. Western blot analysis was preformed (figure 2a-d). All 3 inhibitors significantly decreased SREBP-1 activation (figure 2a).

2.1.2 **Inhibition of SREBP-1 down regulates fatty acid and cholesterol synthesis in GBM tumor cells.**

Fatty acid synthase (FAS), Stearoyl-CoA desaturase-1(SCD-1), and HMG-CoA reductase (HMGCoAR) are important lipogenic genes regulated by SREBPs. FAS is an enzyme that mainly catalyzes the synthesis of palmitate from acetyl CoA and malonyl CoA; whereas SCD-1 catalyzes the rate-limiting step in the formation of monounsaturated fatty acids (MUFAs). HMGCoAR is an enzyme regulating the rate-limiting step in cholesterol synthesis.

In order to investigate the effect of SREBP1 inhibition on these three main lipogenic genes, we measured the protein expression levels of FAS, SCD-1, and HMGCoAR in T98G tumor cells treated with the inhibitors. The T98G cells were incubated with 250 μM Indip (INDIP), 5 μM fatostatin (FAT), 10 μM PF429242 (PF) for 48h then analyzed for FAS by Western blot (Fig. 2b). All the three inhibitors induced a decrease (20 % to 75%) in FAS expression (Fig. 2b). PF429242 significantly reduced (45%, $p<0.001$, n=3) HMGCoAR expression compared to the control (C) (Fig. 2c). The

effects of Fatostatin and Indip on HMGCoAR reduction did not reach to a significant level (Fig. 2c). As shown in Fig 2d, PF429242 drastically decreased SCD-1 expression (80%, $p<0.001$, n=3) whereas Indip and fatostatin reduced the expression by (40-50%) compared to that of the control (Fig. 2d). These data show that all three SREBP-1 inhibitors inhibited expression of both FAS and SCD-1; only treatment with PF429242 resulted in decreasing expression of HMGCoAR.

2.1.3 **Inhibition of SREBP-1 causes G_2/M cell cycle arrest and caspase-mediated apoptosis in the glioblastoma cell line T98G.**

To determine the percentage of cells that were arrested at the G_2/M cell cycle point, T98G cells were incubated in 10%-FBS-EMEM alone or with 5 μM fatostatin (FAT), 10 μM PF429242 (PF) and 250μM Indip (INDIP) for 24, 48 and 72 hrs at 37ºC and 5% CO_2. At the appropriate time point, cells were fixed then incubated in propidium iodine to determine cell cycle distribution by flow cytometry. The percent of cells in the G_0/G_1, S and G_2/M phase was determined for each time point (figure 3a-c). In the untreated cells (c) only 5.5% $\pm$ 1% were arrested in the G_2/M phase (figure 3d). Treatment of the cells with FAT showed the most significant increase in cells arrested in the G_2/M phase (23%) compared to control (c) at 48 hrs (figure 3d). Treatment of the cells with Indip showed the lowest percent of cells (13%) at the G_2/M phase which is an 7% increase in cells arrested in the G_2/M phase compared to control (figure 3d). Treatment with PF429242 had an (6-12%) increase in cells arrested at the G_2/M phase at the 48hr time point and 2.25-fold increase at 72hrs compared to control (figure 3d). These data show that treatment of T98G cells with SREBP-1 inhibitors decrease cell proliferation and this decrease is due impart to G_2/M cell cycle arrest.

It has been shown in prostate cancer cells that fatostatin induces apoptosis through the activation of caspase-3[17]. We therefore investigated whether the inhibition of SREBP-1 similarly induces apoptosis in T98G cells. Cells were incubated with 250 μM Indip (INDIP), 5 μM fatostatin (FAT), 10 μM PF429242 (PF) for 24, 48 and 72 hrs. The cells were then measured by flow cytometry-based propidium iodine (PI) staining. Treatment of T98G cells with both fatostatin and PF429242 induced significant cell death (18-40%, $p<0.001$, n=3) at 48 and 72 h (Fig. 3e). Whereas, Indip-treated cells only had a small effect on apoptosis compared to fatostatin and PF429242. The apoptotic effects of fatostatin, PF429242 and Indip appeared to be time-dependent. These data indicate that inhibition of SREBP-1 promotes apoptosis in glioblastoma cells.

To further define the underlying mechanism for apoptotic cell death, we looked at caspase activation. Cells were treated with 250 μM Indip (INDIP), 5 μM fatostatin (FAT), 10 μM PF429242 (PF) for 48 h and then immunoblotted for active caspase-3 (Fig. 3f). Quantitative analysis showed that both PF429242 and fatostatin caused a significant increase in caspase-3 activation compared to untreated cells (control) (Fig. 3f). Treatment with fatostatin and PF459245 increased caspase activity by 2.9 and 3.7 fold ($p<0.001$, n=3), respectively whereas Indip treatment had no significant effect. Therefore, the inhibition of SREBP-1 triggers the apoptotic cascade pathway at least in part by caspase-3 activation in glioblastoma cells.

2.1.4 **Treatment of T98G glioblastoma cells with SREBP-1 inhibitors modulates cell morphology and inhibits Multicellular Tumor Spheroids (MCTS) growth.**

It has been shown that T98G cells are a hyperpentaploid cell line with irregularly shaped nuclei and are larger in size than normal astrocytes[18]. In order to determine if inhibition of SREBP-1

would affect cell morphology and inhibit the growth of tumor spheres, T98G cells were treated with control (C) or 5 μM fatostatin (FAT), 10 μM PF429242 (PF) and 250 μM Indip peptide (INDIP) for 72 h at 37°C and 5%CO_2. The treated cells were then detached with trypsin and imaged using phase contrast microscope. The untreated cells (C) contained a significant number of giant cells (Fig. 4a). Quantitative analysis of cell size revealed that the treatment of T98G cells with Indip or fatostatin significantly decreased the relative cell size by 35% and 42% respectively (Fig. 4b). Although compared to both fatostatin and Indip, the treatment with PF429242 resulted in smaller but significant reduction (16%) in cell size (Fig. 4B), however, PF429242 altered T98G cell morphology (Fig. 4A).

We also investigated the ability of indip, fatostatin, PF429242 and temozolomide to inhibit the growth of MCST. T98G cells were grown in Matrigel as indicated in Materials and Methods to form tumor spheres[19]. In brief, T98G cells were suspended in Matrigel (5.0 x 10^6 cells/ml) then 1.2 ml was plated in each well of a 6-well tissue culture plate and incubated at 37°C for 30 min. The cells were further incubated in media alone or with 250 μM Indip (INDIP), 5 μM fatostatin (FAT), 10 μM PF429242 (PF) and 500 μM temozolomide (TMZ) then incubated for 7 and 14 days at 37°C and 5% CO_2. The cells were imaged by phase contrast microscope (Fig. 4c). Quantitative analysis showed that at day 7, fatostatin, PF429242 and TMZ drug treatments had a similar decrease in MCTS size (>35%) compared to untreated cells (C). In contrast, Indip had minor effects on inhibiting the growth of MCST (Fig. 4d). At day 14, the MCST treated with PF429242 showed a significant decrease (80%, $p< 0.001$, n=3) in tumor size compared to control (Fig. 4c-4d). Both TMZ and fatostatin showed a significant decrease in tumor sphere size (60% and 40% respectively, $p< 0.001$, n=3) compared to control. However, Indip failed to inhibit the growth of

MCST (Fig. 4c-4d). Taken together these data indicate that inhibition of SREBP-1 reduces the growth of the T98G tumor sphere through in part by inhibiting cell proliferation. These data indicate that SREBP-1 inhibition can significantly inhibit glioblastoma growth.

2.1.5 Inhibition of SREBP-1 impedes glioblastoma cell migration through down regulation of CDK-5 expression.

The ability of progressive and metastatic cancer cells to migrate into distant regions and tissues is one of the hallmarks to disease progression. The effects of fatostatin, PF429242 and Indip on the migration of T98G cells was determined using a scratch assay. A denuded area was made in the T98G monolayer. The cells were incubated in media alone or with 250 μM Indip (INDIP), 5 μM fatostatin (FAT) and 10 μM PF429242 (PF) for 72 h at 37°C and 5% CO_2. Images were taken at 6-hour intervals from zero to 72 h. The migration rate of the cells into the denuded region was determined. After 72 h treatment with PF429242 a significant reduction ($p< 0.001$, $n=3$) in cell migration rate was observed and the cells treated with PF429242 had a 76% decrease in migration rate compared to controls (C) (Fig. 5a-5b). Treatment with fatostatin and Indip resulted in 57% and 25% decrease in migration rate, respectively (Fig. 5a-5b).

It appears that cyclin dependent kinase 5 (CDK-5) expression is regulated by SREBP-1 and involved in T98G cell migration. Cyclin dependent kinases (CDK) are well known to play a role in cell migration and have been identified to be involved in promoting metastasis by reducing the activity of caldesmon[20] thus making CDKs a target for glioblastoma therapy[21]. These studies linking CDK-5 as a possible key mediator in cancer cell migration and metastasis led us to investigate whether the inhibition of SREBP-1 could affect CDK-5 protein expression. Moreover,

the inhibition or knockdown of CDK-5 have been shown to have an anticancer effect and also synergizes the effectiveness of chemotherapeutic agents [22]. T98G cells incubated with 250 μM Indip (INDIP), 5 μM fatostatin (FAT) and 10 μM PF429242 (PF) were analyzed for CDK-5 protein expression by Western blot (Fig. 5c). Treatment of T98G cells with PF429242 showed a significant decrease (55% $p<0.001$, $n=3$) in CDK-5 expression compared to untreated cells (C) (Fig. 5c). In contrast, both fatostatin and Indip had minor effects on CDK-5 protein expression. This observation suggests that the inhibition of SREBP-1 down regulates the expression of CDK-5 and may play a role in the mechanism whereby SREBP-1 inhibitors prevent cancer cell migration. This finding is the first to show that CDK-5 expression is regulated by SREBP-1 activity. Blast analysis revealed that potential sterol regulatory element (SRE) and EBOX sequences were found at 2-9 and 3-9 positions of CDK-5 gene, respectively, with 100% homology/similarity which warrants further investigation.

2.1.6 Combined direct and indirect SREBP1 inhibitors enhanced T98G tumor cell death compared to individual treatment.

Most cancers occur due to a heterogeneous biology which includes multiple genetic change or abnormalities, disruption of multiple signaling pathways and cellular heterogeneity[31]. Therefore, it has been observed that single drug therapy is not sufficient to treat cancer effectively[32]. Presently, combination drug regimens are a standard strategy to maximize drug efficacy and to reduce cytotoxicity for normal cells [33, 34]. Therefore, we investigated whether combinations of SREBP-1 inhibitors produce a synergistic or an additive effect in promoting apoptosis. T98G cells were incubated with different combination of 250 μM Indip (INDIP), 5 μM fatostatin (FAT), 10

μM PF429242 (PF) and 500 μM temozolomide (TMZ) for 24, 48 and 72 h and then analyzed for viability. As shown in Fig. 6, all combinational treatment resulted in enhanced cell death when compared to their respective individual treatment. The combination treatment with Indip had the greatest effect on promoting cell death (Fig. 6a-6c). Indip/PF429242 treatment caused 87.5% tumor cell death. This was a 5-fold increase compared to Indip alone and a 3-fold increase compared to PF429242 alone (Fig. 6c). The combinations of Indip/TMZ and Indip/fatostatin were also effective in inducing cell death compared to the individual compounds (37% and 15%, respectively) (Fig. 6c). In addition, combination of PF429242/fatostatin and PF429242/TMZ has similar percentage of apoptosis (Fig. 6f). Similar results for the combination treatment were also observed at 24 and 48 h time points (Fig. 6a, 6b, 6d and 6e). Collectively, these results indicate that combination therapy may be more efficient at eliciting an apoptotic effect than the individual treatment. Moreover, inhibition of Insig-1 degradation and inhibition SREBP-1 activity additively exhibits the greatest efficacy for inducing apoptosis. These findings provide a rationale for the development of novel therapies for GBM.

2.2.1

Figure 1.

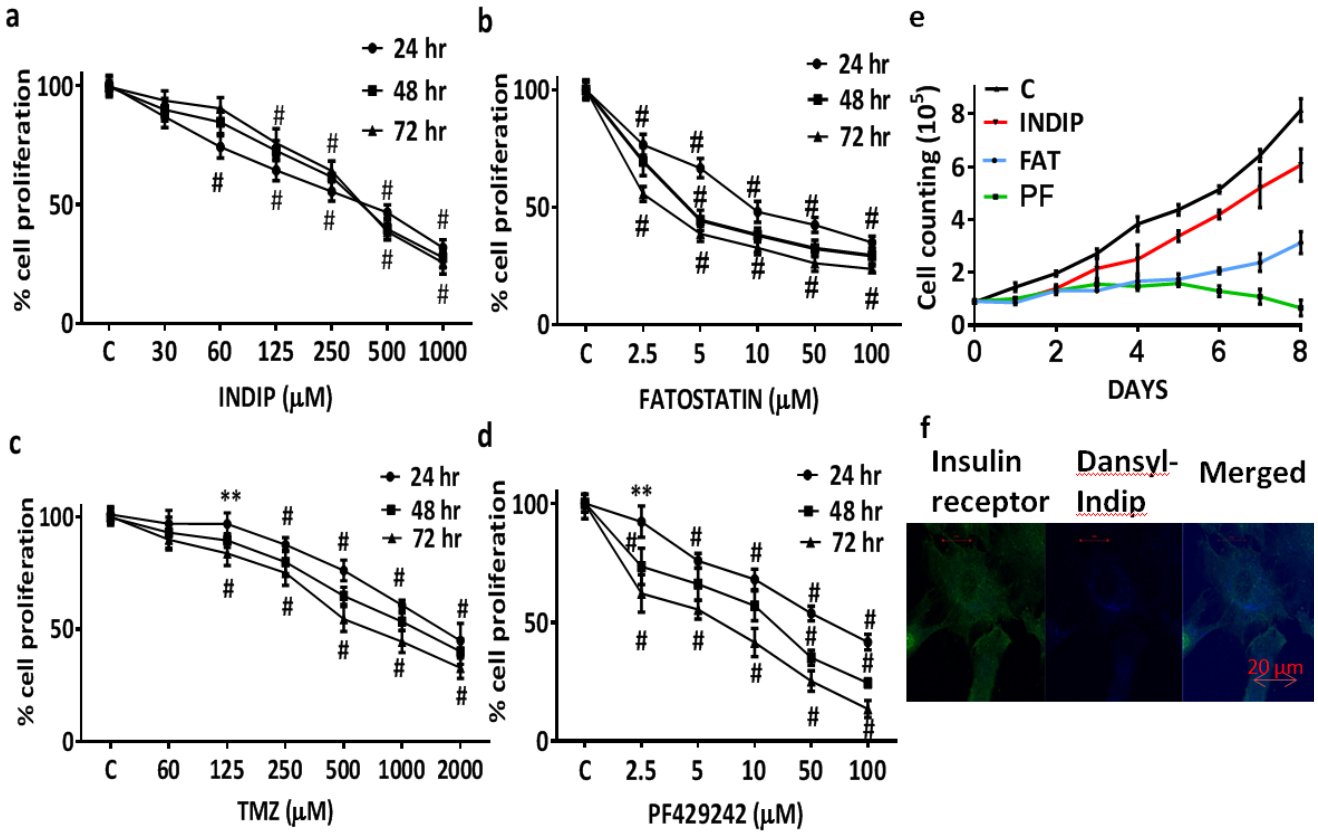


Figure.1 Time and concentration-dependent effects of Indip, fatostatin, PF429242 and Temozolamide (TMZ) on percentage (%) cell proliferation in T98G cells.

Indip, Fatostatin, PF429242 and Temozolamide inhibited the proliferation of T98G cells in a time- and dose-dependent manner. T98G cells were incubated with increasing concentrations of inhibitor, incubated for 24 h, 48 h, and 72 h and then cells were assayed for proliferation using the CCK-8 assay. The values are represented as percentage cell proliferation. where the vehicular control groups (C) were considered as 100%, (a) Indip (0 to 1,000 μM), (b) fatostatin (0 to 100 μM), (c) Temozolomide (0 to 2,000 μM), (d) PF429242 (0 to 100 μM). (e) Fatostatin and PF429242 significantly impeded proliferation of T98G cells over time. Treatment with Indip had only a minor

effect on inhibiting cell proliferation after 4 days. T98G cells were incubated with 250 µM Indip (INDIP), Fatostatin 5 µM (FAT) and PF429242 10 µM (PF) for 8 days. Proliferation was determined by cell count. (f) T98G cells were incubated with dansyl fluorophore labeled Indip for 2 hrs and then incubated with anti-inulin receptor antibody, followed by incubating with Alexa Fluor conjugated secondary antibody for fluorescent. Dansyl Fluorophore labeled Indip is shown in blue and insulin receptor reference in green. Images were taken using laser scanning microscopy with 100X magnification. Each point represents mean ± SEM (n=3) values. The values were measured using one way Anova followed by tukey's multiple comparison test as post hoc in Graphpad Prism version 6.0. ** and # indicates *P* value < 0.01 and 0.001, respectively vs vehicular control. Each point represents mean ± SEM (n=3) values. The values were measured using one way Anova followed by tukey's multiple comparison test as post hoc in Graphpad Prism version 6.0. ** and # indicates *P* value < 0.01 and 0.001, respectively vs vehicle control.

2.2.2

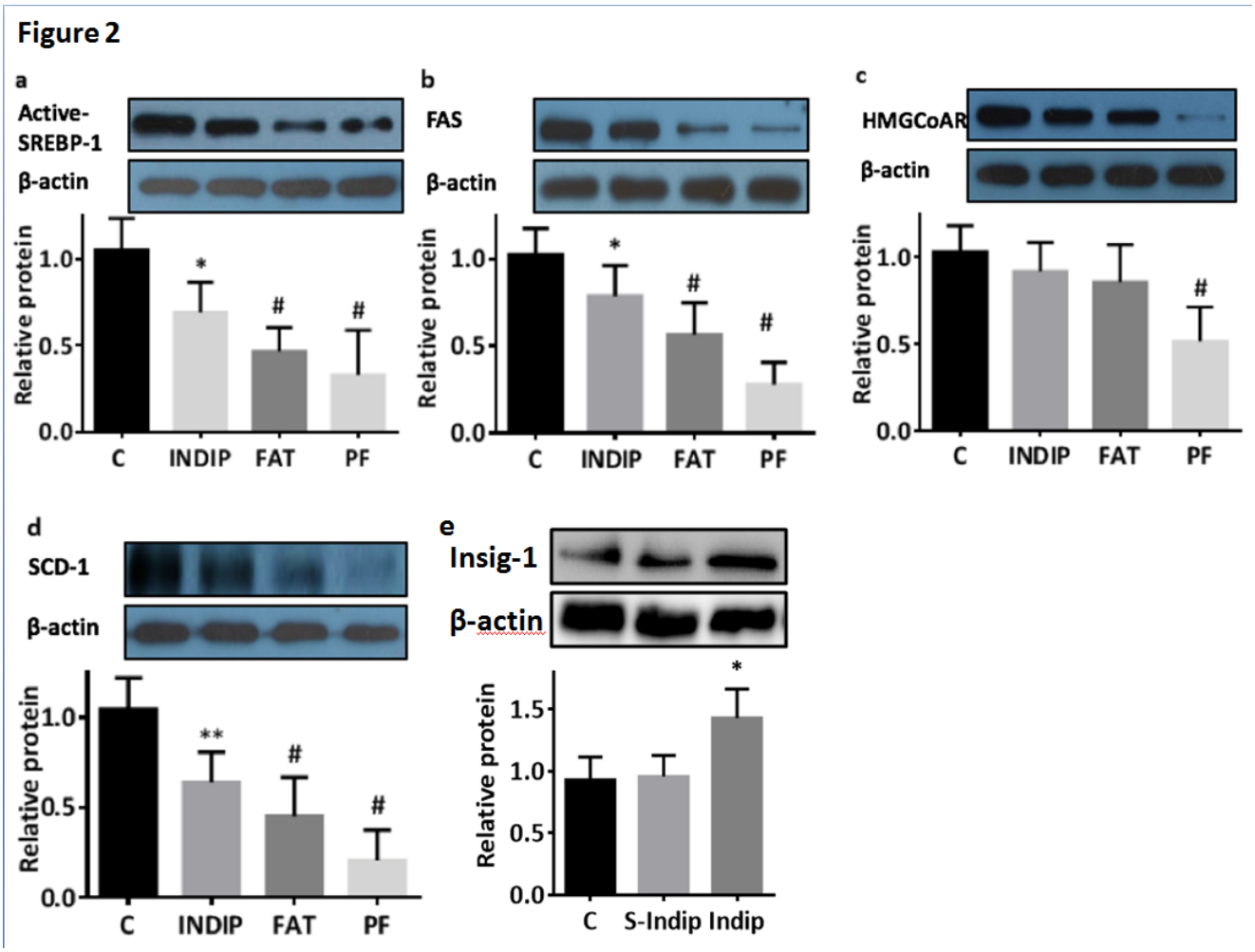


Figure.2 Inhibition of SREBP-1 reduces fatty acid synthesis by hindering SCD-1 and HMGCoAR expression.

Western blot analysis of sterol regulatory element binding protein 1 (SREBP1) and its downstream signaling proteins on 250 µM Indip (INDIP), 5 µM Fatostatin (FAT) and 10 µM PF429242 (PF) exposed T98G glioblastoma cells after 48 h incubation. Untreated cells were incubated without inhibitors (C). Representative western blots of SREBP1 and its downstream signaling proteins along with loading control (β-actin) were shown. (a) ACTIVE-SREBP-1, (b) fatty

acid synthase, (c) Hydroxymethylglutaryl-CoA reductase (HMGCoAR), (d) Stearoyl-CoA desaturase (SCD-1), (e) Insulin induced gene-1 (Insig-1). Values were expressed in mean±SEM, n=3 and similar results were obtained in three individual experiments. Mean difference between the exposure time was measured using one-way ANOVA followed by Tukey's multiple comparison test as post hoc in Graphpad Prism version 6.0. *, ** and # indicates *P* value < 0.05, 0.01 and 0.001, respectively vs control (C).

2.2.3

Figure 3

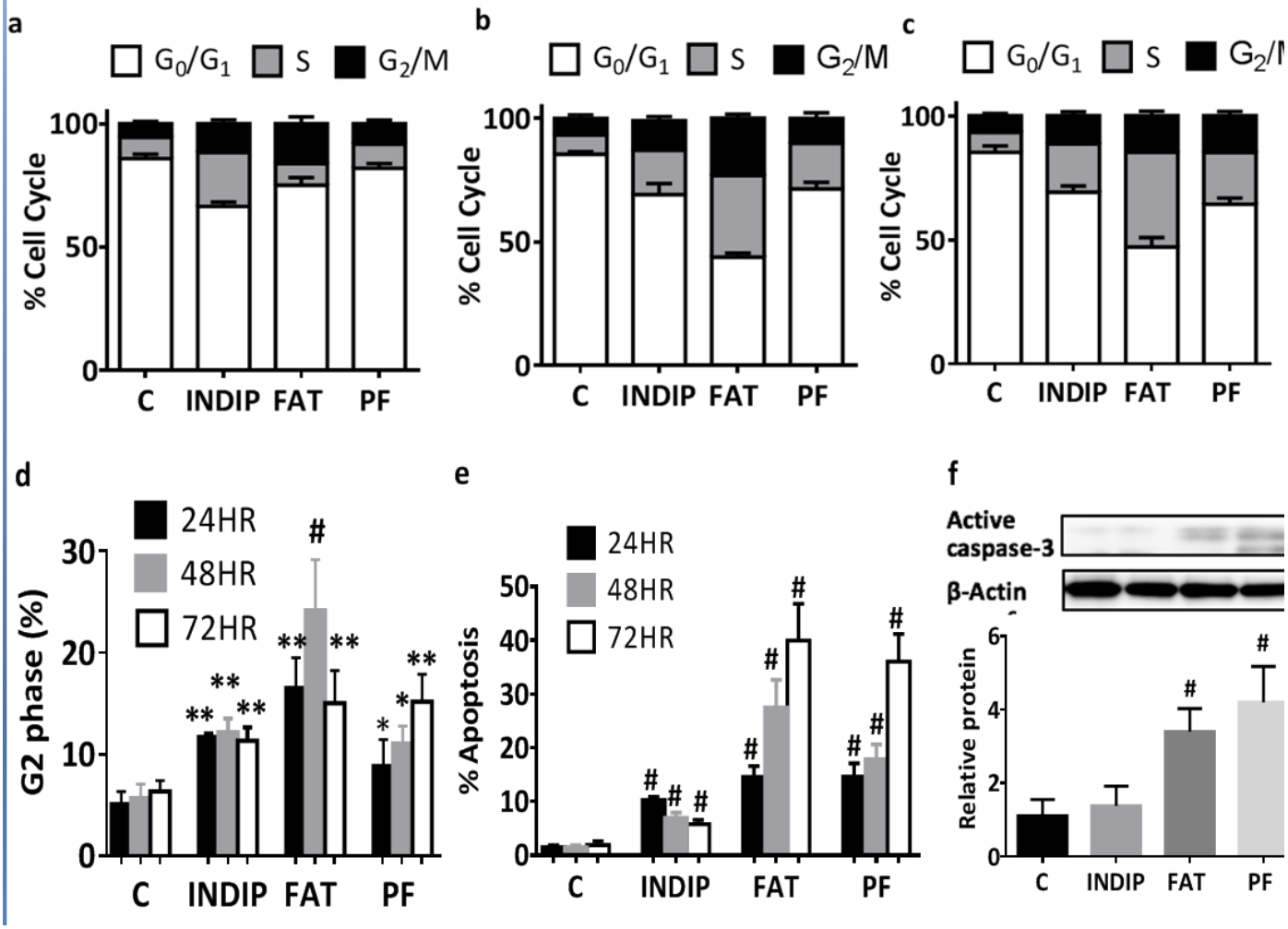


Figure.3 Inhibition of SREBP-1 promoted cell cycle arrest in the G_2/M phase and induced caspase-mediated apoptosis in glioblastoma cancer cells.

Cells were incubated with 250 µM Indip (INDIP), 5 µM Fatostatin (FAT) and 10 µM PF429242 (PF) for 24 h, 48 h and 72 h. Cell cycle phase was determined by flow cytometry based PI-staining and quantitated by FloJo software. Graph represents percentage cell cycle arrest (G2/M, S and G1/M phase cell cycle) of Indip, fatostatin and PF429242 at 24 h (a), 48 h (b) and 72 h (c), respectively. (d), Determination of G_2/M phase arrest in T98G cells on exposure with 250 µM Indip (INDIP), 5 µM Fatostatin (FAT) and 10 µM PF429242 (PF) at various time periods. (e), 250 µM Indip (INDIP), 5 µM Fatostatin (FAT) and 10 µM PF429242 (PF) induce apoptotic cell death in T98G glioblastoma cells. Percentage of apoptotic cell death was measured at various time points using flow cytometry based PI-staining and quantitated by FloJo software. (f), Caspase-3 activity was determined by western blot. 5 µM Fatostatin (FAT) and 10 µM PF429242 (PF) increased active-caspase expression by 2.9 and 3.7 fold, respectively. However, treatment with 250 µM Indip (INDIP)had no significant effect on activating caspase-3. Values were expressed in mean±SEM, n=3 and similar results were obtained in three individual experiments. Mean difference between the exposure time was measured using one way ANOVA followed by tukey's multiple comparison test as post hoc in Graphpad Prism version 6.0. *, ** and # indicates *P* value < 0.05, 0.01 and 0.001, respectively vs control.

2.2.4

Figure 4

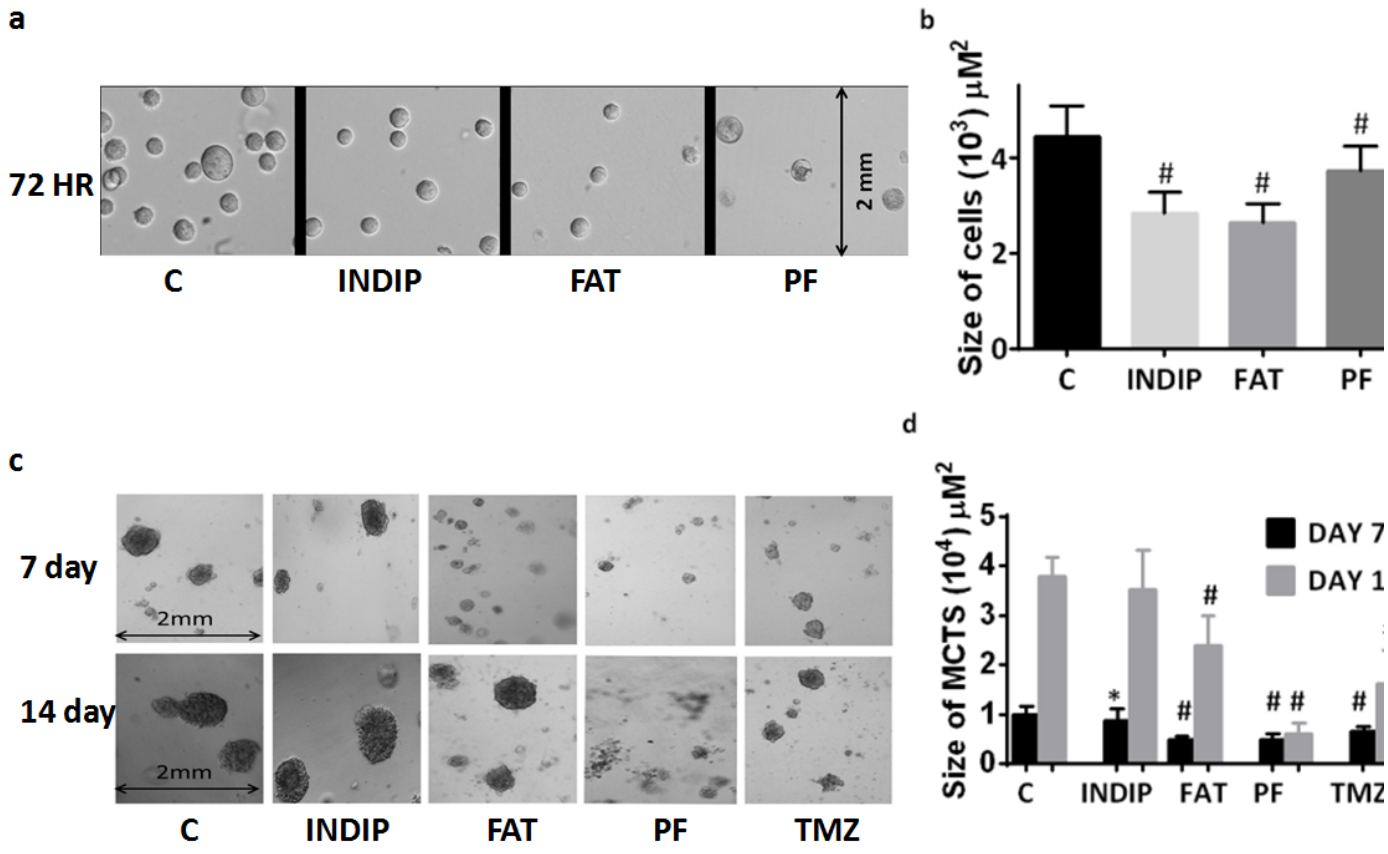


Figure.4 Evaluation of cytotoxicity on cell size and multicellular tumor spheroid (MCTS)

Inhibition of SREBP-1 reduced cell size and MCTS growth. T98G cells were incubated with 250 µM Indip (INDIP), 5 µM Fatostatin (FAT) and 10 µM PF429242 (PF) for 72hrs and then the diameter of each T98G cell was measured by Phase-contrast microscopy at 10x magnification after detaching the cells with trypsin and 2D surface area of cells were quantified. (a), T98G cells images produced by Phase-contrast microscopy after 72 hr incubation Indip, (b), Quantitative

analysis of the cell size after incubating with the SREBP inhibitors. Indip, Fatostatin, PF429242 and Temozolomide impeded MCTS formation and growth in T98G cells. (c), The formation of MCTS from T98G cells after 7 and 14 days incubation. A phase-contrast microscopy was used to monitor the MCTS formation and growth. (b), The diameter of each MCTS was measured by phase contrast microscopy at 4X magnification and 2D surface area of MCTS was calculated. T98G cells were cultured in EMEM with Matrigel and then treated with 250 µM Indip (INDIP), 5 µM Fatostatin (FAT), 10 µM PF429242 (PF) and 500 µM Temozolomide (TMZ) for 7 and 14 days. Values were expressed in mean±SEM, n=3 and similar results were obtained in three individual experiments. Mean difference between the exposure time was measured using one-way ANOVA followed by Tukey's multiple comparison test as post hoc in Graphpad Prism version 6.0. * and # indicates *P* value < 0.05 and 0.001, respectively vs control.

2.2.5

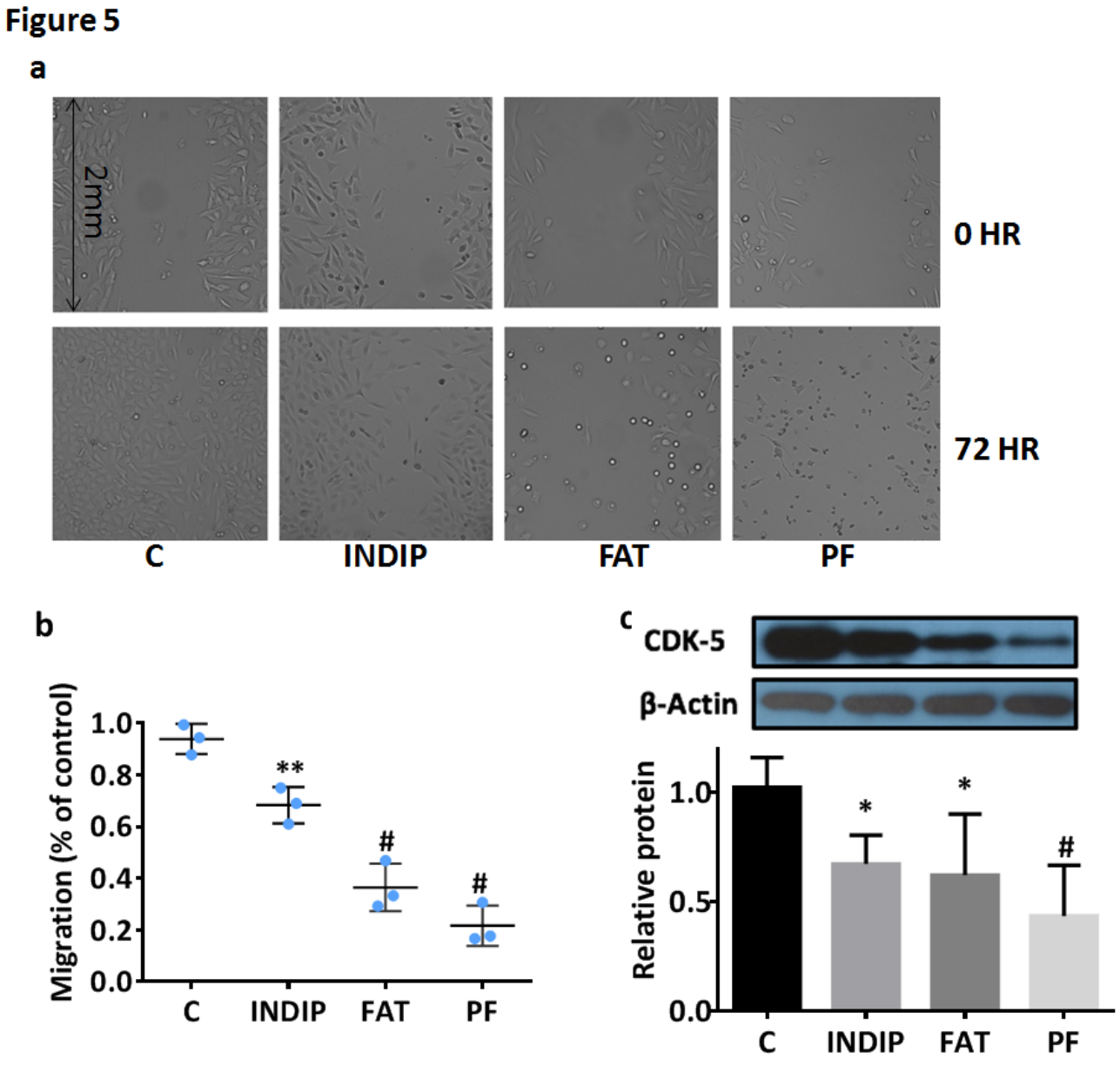


Figure. 5 SREBP-1 inhibition significantly impeded the ability of T98G cells to migrate

Determination of the relative migration rate (distance traveled) by T98G cells into the acellular region on 250 µM Indip (INDIP), 5 µM Fatostatin (FAT) and 10 µM PF429242 (PF) exposure using scratch assay. (a) Cell migration was photographed at 0 and 72 h later. (b) Quantitative analysis

of the cellular migration rate after 72 h. (c) The decrease in T98G migration was through inhibition of CDK-5. Cells were treated with 250 µM Indip (INDIP), 5 µM Fatostatin (FAT) and 10 µM PF429242 (PF) for 48 h then Western blotted for CDK-5. Untreated cells were incubated without inhibitors (C). Values were expressed in mean±SEM, n=3 and similar results were obtained in three individual experiments. Mean difference between the exposure time was measured using one way ANOVA followed by Tukey's multiple comparison test as post hoc in Graphpad Prism version 6.0. *,** and # indicates *P* value < 0.05, 0.01 and 0.001, respectively vs control.

2.2.6

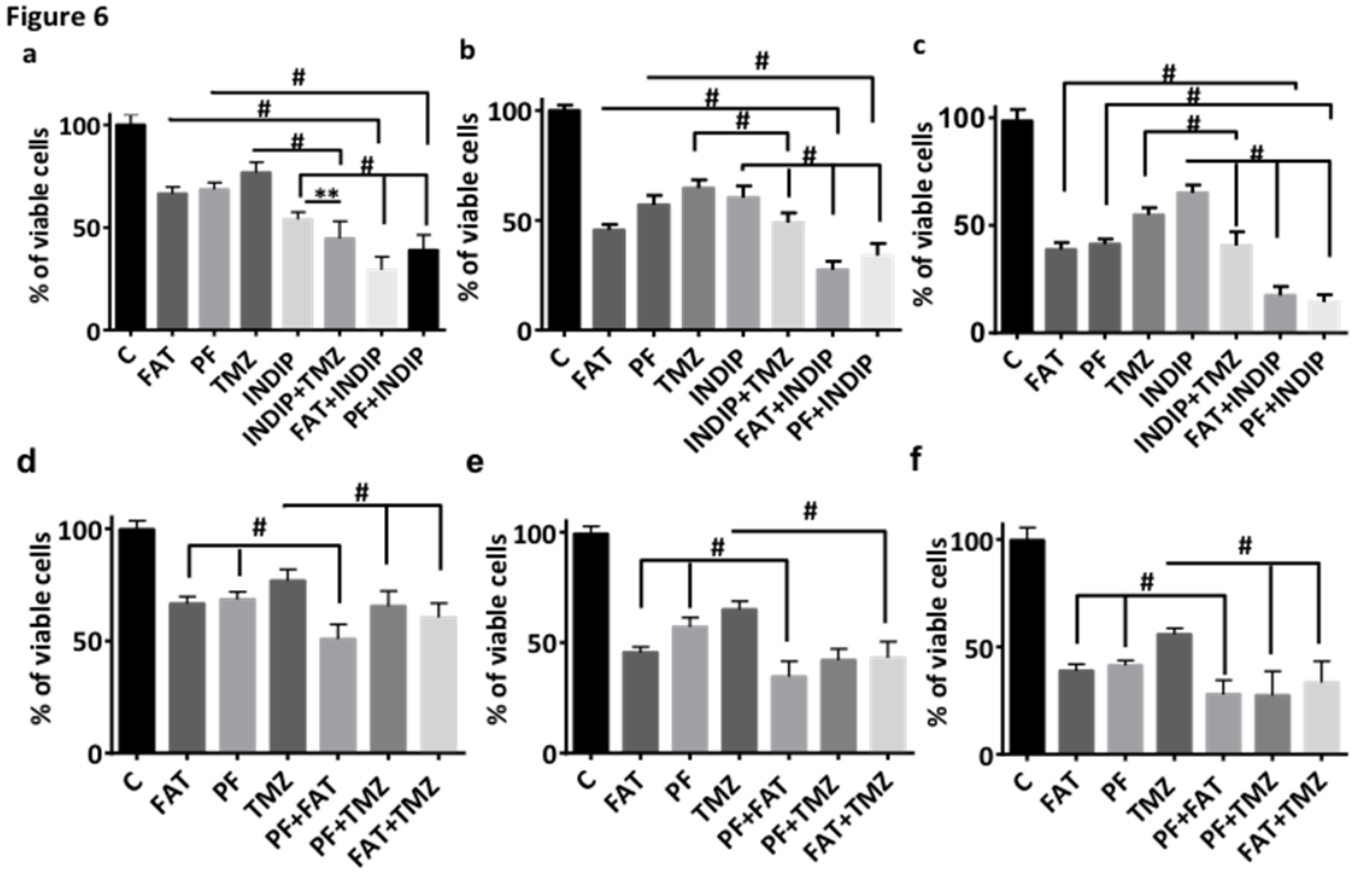


Fig.6. Treatment with Indip, fatostatin, PF429242 and TMZ have an additive effect on suppressing proliferation of T98G cells.

Cytotoxicity assay of drug combinations 250 µM Indip (INDIP), 5 µM Fatostatin (FAT), 10 µM PF429242 (PF) and 500 µM Temozolomide (TMZ) in T98G cells. The cells were then analyzed for cell death by CCK-8 assay. Graph represents the percentage viable cells at (a) 24 h, (b) 48 h, (c) 72 h, (d) 24 h, (e) 48 h and (f) 72 h exposure period. Values were expressed in mean±SEM, n=3 and similar results were obtained in three individual experiments. Mean difference between the exposure time was measured using one way anova followed by tukey's multiple comparison test as post hoc in Graphpad Prism version 6.0. *,** and # indicates *P* value < 0.05, 0.01 and 0.001, respectively vs control.

2.2.7

Figure 7

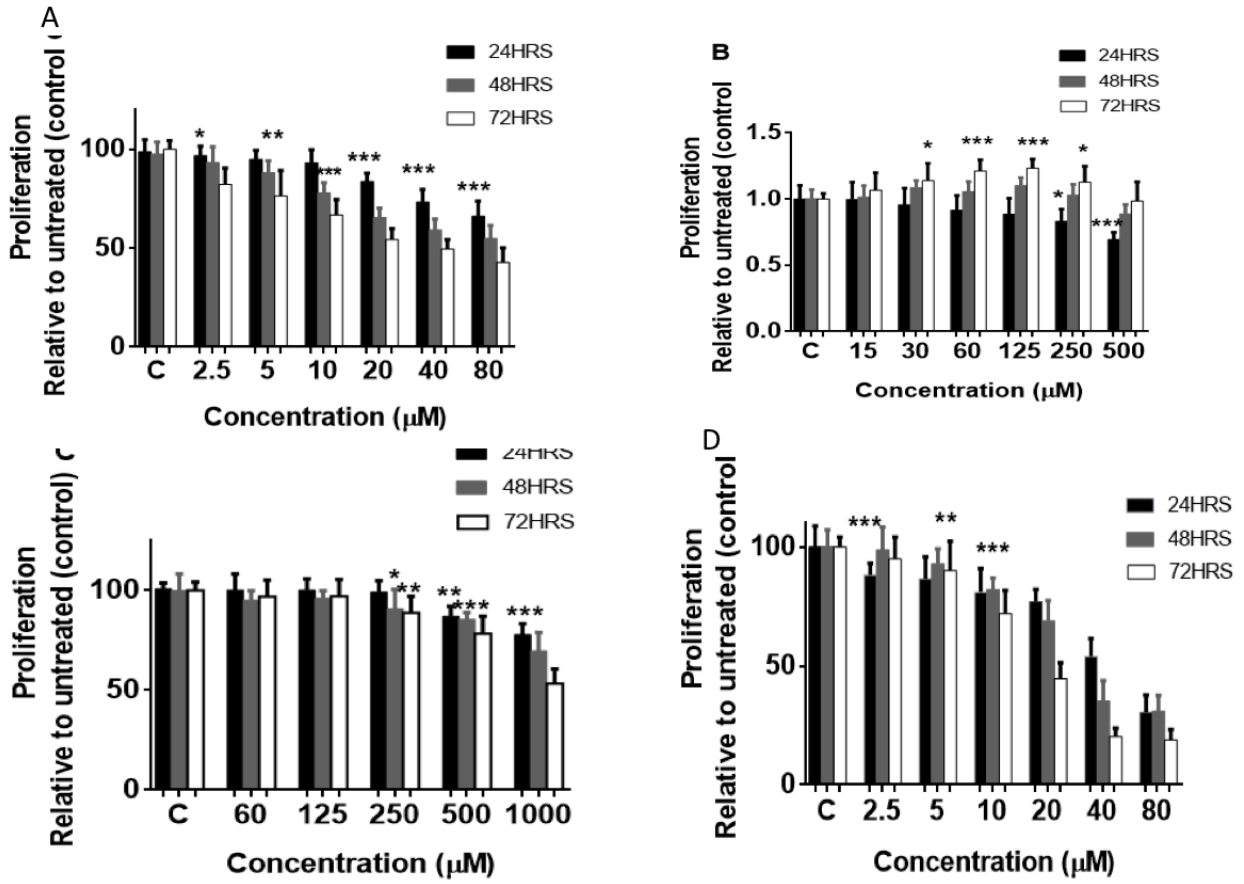


Figure.7 Time and concentration-dependent effects of Indip, fatostatin, PF429242 and Temozolamide (TMZ) on percentage (%) cell proliferation in the rat glial cells.

Indip, Fatostatin, PF429242 and Temozolamide inhibited the proliferation of rat glial cells in a time- and dose-dependent manner. Rat glial cells were incubated with increasing concentrations of inhibitor, incubated for 24 h, 48 h, and 72 h and then cells were assayed for proliferation using the CCK-8 assay. The values are represented as percentage cell proliferation. where the vehicular control groups (C) were considered as 100%, (a) Indip (0 to 500 μM), (b) fatostatin (0 to 80μM), (c) Temozolomide (0 to 1,000 μM), (d) PF429242 (0 to 80 μM). Each point represents mean ± SEM (n=3) values. The values were measured using one way Anova followed by tukey's multiple

comparison test as post hoc in Graphpad Prism version 6.0. ** and # indicates *P* value < 0.01 and 0.001, respectively vs vehicular control. Each point represents mean ± SEM (n=3) values. The values were measured using one way Anova followed by tukey's multiple comparison test as post hoc in Graphpad Prism version 6.0. ** and # indicates *P* value < 0.01 and 0.001, respectively vs vehicle control.

MATERIALS AND METHODS

Material and Methods

2.3.1 Cell Culture.

T98G Glioblastoma cell lines were obtained from American Type Culture Collection (ATCC® CRL-1690™). Cells were grown as monolayer in cell culture flasks with EMEM media (HyClone, Cat# SH30024.01) containing 10% (v/v) fetal bovine serum (FBS) (HyClone, Cat#SH30070.01)

2.3.2 Cytotoxicity.

Cytotoxicity assay of T98G cells was performed by using CCK-8 Assay kit (Dojindo: CK04-11) as indicated by manufactures protocol. Mono layers of T98G cells were grown in 10% FBS-EMEM in 96-well plate (5,000 cells/well) overnight at 37°C and 5%CO_2. The media was removed and replace with 100 ml of 10% FBS-EMEM media with or without without Indip (BIOMATIK, Cat # 11290), fatostatin (TOCRIS BIOSCIENCE, Cat # 4444), PF429242 (Sigma, Cat # SML0667) or TMZ (Sigma, Cat # T2577) as indicated in results and incubated at 37°C and 5%CO_2. The cells were

incubated at various time intervals (24h, 48h and 72h), then 10 µl of CCK-8 solution was added to each well and incubated for 2h at 37°C and 5%CO_2. The samples were analyzed for soluble formazan produced using a SpectraMax M5 plate reader at 450 nm (Molecular Devices). All samples were performed in triplicate. The mean of each sample was determined.

2.3.3 **Western blotting.**

T98G cells were plated in 10 cm petri dishes and incubated in 10% FBS-EMEM media at 37°C and 5%CO_2. After overnight attachment, the cells were treated with either vehicle (control), PF429242, Indip or fatostatin for 48 hours. The concentrations of fatostatin, Indip and PF429242 employed were chosen based on the IC_{50} values derived from preliminary studies. For total protein extraction, cells were lysed in RIPA buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM β-glyceroposphate, 1 mM Na_3VO_4, 1 µg/ml leupeptin) supplemented with protease inhibitor cocktail (Cell Signaling, #5871). Total protein extracts were quantified using the Bradford assay with bovine serum albumin as standard. Western blot experiments were carried out using the Mini Trans-Blot cell BIO-RAD. The following primary antibodies were used: anti-SCD1 (Abcam, ab19862; dilution 1:1000), anti-CDK5 (Abcam, ab715Y; dilution 1:1000), anti- HMGCoAR (Abcam, ab174830; dilution 1:1000), anti-SREBP1 (Santa Cruz, sc-365513, 1:500), anti-fatty acid synthesis (Abcam, ab22759; dilution 1:1000) and anti-caspase-3 (Cell signalling, #9662; dilution 1:1000) Anti-β actin (Abcam, ab8227; dilution 1:1000) was used for normalization. All the experiments were performed in triplicate.

2.3.4 Flow cytometry.

Cells were seeded in 6-well plates (1 X 10^5 cells/well), incubated overnight in 10% FBS-EMEM media at 37ºC and 5%CO_2. The media was removed and replaced with 10% FBS-EMEM media containing SREBP-1 inhibitors as indicated in the results section. The cells were further incubated at 37°C and 5%CO_2 for 24, 48 and 72 hrs. At the specified time points, cells were trypsinized, harvested, fixed in ice cold 70% ethanol-PBS for 10 min and then stored at 4ºC until further use. For analysis, the cells were removed from the -20ºC storage and incubated on ice for 15 min. Cells were washed in ice cold PBS X 2 times and then incubated in staining buffer (60 µg/ml propidium iodide; Sigma-P4170 and 0.15 mg/ml RNase A; Sigma-R6513) for 20 min. For each sample ~1 million gated cells were analyzed by the flow cytometry (BD-FAC Scalibur^{TM}). Data analysis was preformed using FlowJo software (FlowJo, LLC). All samples were run in triplicate.

2.3.5 **Multicellular Tumor Spheroids (MCTS) culture and analysis:**

The MCTS culture has been adapted from Lee et al[20]. In brief, coating of the 6 well plates was performed by adding 120 µl/well of Matrigel (Sigma, E1270), then spreading evenly on the surface with a pipette tip. The coated plates were incubated at 37°C for 15 min. T98G Glioblastoma cells were trypsinized into a single cell suspension. The cells were centrifuged at 115 x g for 5 min at 4ºC and then the supernatant was removed. The cells were resuspended in ice cold Matrigel (5.0 x 10^6 cells/ml). The T98G cells were plated on Matrigel coated 6-well TC plates (1.2 ml/well) and incubated at 37ºC for 30 min. To each well, 3 ml of 10% FBS-EMEM media was added with or without inhibitors as indicated in results. The cells were incubated for 14 days at 37°C and 5%CO_2 and the media was changed every 2 days. At day 7 and 14, the tumor spheres

were imaged on a phase contrast microscopy (OLYMPUS, IX71) at 4X magnification. Tumor sphere size was analyzed using ImageJ software.

2.3.6 **Scratch Assay.**

T98G cells were grown in 10% FBS-EMEM media in a 6 wells plate with or without SREBP-1 inhibitors as indicated in results and incubated at 37°C and 5%CO_2. The monolayer was scrapped with a p200 pipette tip to make a denuded area. The debris was removed and the cells were washed once with 1 ml of the 10% FBS-EMEM media to remove additional dislodged cells. The cells were then incubated with 10% FBS-EMEM media with inhibitors as indicated in results for 72 h at 37°C and 5%CO_2. Each flask was marked so that the same reference area was imaged up to 72 h with 6 h interval. The denuded area was imaged using a phase contrast microscope using a 4x objective lens. The relative distance traveled by the cells into the acellular region was determined using ImageJ software. Cells treated with diluent were used as a baseline control.

2.3.7 Immunofluorescence staining and Fluorescence Studies of Labeled Indip Containing Dansyl Fluorophore.

Immunostaining was adopted from Abcam protocol. Briefly, coverslips were coated with polyethylineimine for 5 min at room temperature. Then T98G cells were cultured on glass coverslips at 37°C and 5% CO_2 after rinsing the coverslips in PBS three times. After overnight incubation, T98G cells were incubated with 5-*N*,*N*-dimethylaminonaphthalene sulfonyl (dansyl) fluorophore labelled Indip (BIOMATIK, Cat # 523525) for 2 h. Next, the cells were fixed in 10% formalin for 10 min after briefly washing three times in PBS. To block unspecific epitopes, T98G cells were incubated with 2% BSA, 23mg/mL glycine in PBST for 1 h. Then the cells were incubated

in the diluted primary antibody in 2% BSA (in PBST) for overnight at 4 ^{o}C (Anti-Insulin, Abcam, ab18147; dilution 1: 500), followed by incubating with anti-rabbit ALexa Fluor 488 (Abcam, ab150077; dilution 1: 500). Finally, the cell images were analyzed the laser scanning microscopy (ZEISS, LSM 700).

2.3.8 Isolation of mouse/rat astrocyte.

All procedures on these animals were performed in accordance with and after have approved by the institutional animal care and use committee (IACUC) of University of Saskatchewan. Rats were anaesthetized with isoflurane then euthanized by decapitation. The tissue was washed in 70% ethanol. The brain was removed and the meninges were removed from the cerebral cortex. The brain tissue was washed in Hank's balanced salt solution. Tissue digestion was performed by incubating in trypsin-EDTA (Gibco) for 15 min at 37ºC. The digestion was stopped with the addition of 500μl of FBS (HyClone). The cell suspension was centrifuged for 1 min at 300 xg and the supernatant was removed. The cells were resuspended in 10 ml of astrocyte growth media (high glucose-DMEM (HyClone), 10% FBS (HyClone), 1% penicillin/streptomycin (Sigma-Aldrich)) and mixed by pipetting to dissociate the cells. The cell suspension was placed in a T75 flask then incubated at 37ºC and 5%CO_2 for 48 hr. The media was replaced to removed dead cells and repeated every 3 days until the flask was confluent. Once confluent, the flask was placed on an orbital shaker and shaken at 180 rpms for 30 min at 37ºC and 5%CO_2 to remove microglia. The media was removed and replace with astrocyte growth media. The flask was placed back on the rotator an shaken at 240 rpms overnight at 37ºC and 5% CO_2 to remove oligodendrocyte precursor cells. The media was removed and the cells were washed with PBS (Gibco). The

adherent astrocytes were further incubated in astrocyte growth media at 37ºC and 5% CO_2 until confluent.

2.3.9 Statistical Analysis.

All the statistical analyses were conducted using Graphpad Prism 6 software (GraphPad Software, San Diego, CA). Kaplan-Meyer survival curves were compiled using Prism software (GraphPad). Tumor sphere size, cell cycle, proliferation and cell migration were compared by one-way ANOVA test. Additional experiments were analyzed by the unpaired t-test. Data are presented as mean ± standard deviation (SD). $P < 0.05$ was considered statistically significant.

DISCUSSION

The present study clearly showed that active form of SREBP-1 and its downstream signaling proteins were increased in T98G tumor cells. Inhibition of SREBP-1 overactivation by fatostatin, PF429242 or indip treatment resulted in significant reduction of downstream signaling proteins. In addition, these inhibitors decreased GBM cell line tumor size and induced cell cycle arrest at G2/M phase, leading to apoptotic cell death. Further, combination treatment enhanced the anti-proliferative effect of T98G tumor cells. Thus, it is evident that SREBP-1 inhibitors as individual or in combination may be a novel therapeutic intervention in the management of GBM.

SREBP-1 regulates fatty acid biosynthesis. Insig-1 levels raise with the addition of Indip and subsequent inhibition of the activation of the SREBP-1 lipid transcription factor as the transcription factor cannot escape the membrane. We demonstrated that the inhibition of

SREBP-1 resulted in suppression of three major downstream targets: FAS, SCD-1 and HMGCoAR. SREBP-1 regulation of fatty acid synthase and SCD-1 has been observed in breast, pancreatic and prostate cancers and inhibition of SREBP-1 promoted cancer cell death[13,17,35]. Thus, SREBP-1 may prove to be a central mechanism for tumor cell survival. This correlates with our present findings. CDK-5 is a key kinase in neural maturation and migration, regulating the reelin signaling chain and synaptic vesicle exocytosis, which are key processes in neuronal function[23-25]. The decreased expression of CDK-5 in our present study suggest that SREBPs may have direct or indirect regulation of proteins outside the fatty acid pathways and may regulate cell function at multiple levels. This study also shows that blocking SREBP-1 activity in GBM cells promotes apoptosis through activation of the caspase cascade.

SCD-1 and fatty acid synthase are primary rate-limiting enzymes involved in the biosynthesis of long-chain fatty acids. Both SCD-1 and fatty acid synthase play critical roles in the regulation of carcinogenesis, cell proliferation and apoptosis[26,27]. Furthermore, inhibition of SREBP-1 significantly down-regulated fatty acid synthase and SCD-1 levels, indicating that SREBP-1 inhibition regulates cancer-associated fatty acid metabolism, thus inhibiting GBM cancer cell growth. Cancer cells including T98G cells have active signaling networks to meet their high demand for cholesterol[28]. Overexpression of HMGCoAR is involved in the global metabolic transformation of cancer cells and high levels of cholesterol may negatively affect the outcome of chemotherapy[29]. It is also reported that blocking the intracellular supply of cholesterol through inhibition of HMGCoAR pathway resulted in tumor cell death[30]. Indeed, in our study, we demonstrate that inhibiting SREBP-1 activity with PF429242 suppressed expression of HMGCoAR. Fatty acid synthase, SCD-1 and HMGCoAR are three primary enzymes at rate-limiting steps for

biosynthesis of long-chain fatty acids and cholesterol. Their inhibition significantly limits lipid and cholesterol supply and thus, reduces the metabolic resources needed for uncontrolled proliferation and survival of GBM cells. Our data are highly suggestive that inhibiting lipogenesis and cholesterogenesis are key to reducing cancer cell growth and may offer a novel mechanism in developing a successful therapy for GBM.

It is important to monitor and ensure that these chemotherapeutic drugs are not toxic to normal glial cells prior to in Vivo study. In the rat glia data showed that TMZ, fatostatin, PF429242 and Indip inhibits proliferation of normal glial cells to some exstend (Fig. 7). The IC50 values of TMZ are comparable to what is shown with the P98G cells(talble 1 and 2), we assume that TMZ will also promote healthy/normal glial cell death to the same extent. In comparison, Indip, fatostatin and Pf429242 are less cytotoxic to normal glial cells.

In conclusion, our current study showed that SREBP-1 regulates FAS and SCD-1, key regulators of fatty acid synthesis, as well as HMGCoAR, a key regulator of mevolonate pathway which are involved in the global metabolic tumorigenicity of cancer cells including GBM. SREBP-1 has been found to be up-regulated in GBM, making it a putative target. The data presented herein proposes a novel SREBP1-based paradigm in GBM treatment. We demonstrate that SREBP-1 inhibition regulates the expression of CDK-5 in T98G cells and decrease cancer cell migration. Additionally, this study provides evidence for the clinical significance of SREBP-1 inhibition as a novel therapeutic for cancer. Moreover, these studies provide proof-of-principle evidence that targeting SREBP-1 and its regulatory protein Insig-1 may provide a viable therapeutic approach for the treatment of GBM. Future studies should strive to investigate the impact of drug

combination that inhibit multiple regulators of SREBP-1 expression and activation as novel cancer therapeutics.

Table 1

T98G CELL LINE	Drugs	Indip	FATOSTATIN	PF429242	TMZ
	IC50 of 24hr(µM)	250	5	10	500
	IC50 of 48hr (µM)	155	2.64	5.5	334.3
	IC50 of 72hr (µM)	192.4	1.98	3.9	273.2

Table 2

	Drugs	Indip	FATOSTATIN	PF429242	TMZ
	IC50 of 24hr(µM)	320	19.2	23	458.3

Rat Glial Cells	IC50 of 48hr (μM)	N/A	17.4	19	438.4
	IC50 of 72hr (μM)	N/A	14	12.5	423.2

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CHAPTER 3

Sterol regulatory element-binding protein 1 inhibitors decrease pancreatic cancer cell viability and proliferation

ABSTRACT

Sterol regulatory element-binding protein1 (SREBP1) is a key regulatory factor that controls lipid homeostasis. Overactivation of SREBP1 and elevated lipid biogenesis are considered the major characteristics in malignancies of prostate cancer, endometrial cancer, and glioblastoma. However, the impact of SREBP1 activation in the progression of pancreatic cancer has not been explored. The present study examines the effect of suppression of SREBP1 activation by its inhibitors like fatostatin and PF429242 besides analyzing the impact of inhibitory effects on SREBP1 downstream signaling cascade such as fatty acid synthase (FAS), hydroxymethylglutaryl-CoA reductase (HMGCoAR), stearoyl-CoA desaturase-1 (SCD-1), and tumor suppressor protein p53 in MIA PaCa-2 pancreatic cancer cells. Both fatostatin and PF429242 inhibited the growth of MIA PaCa-2 cells in a time and concentration-dependent manner with maximal inhibition attained at 72 h time period with IC_{50} values of 14.5 μM and 24.5 μM respectively. Detailed Western blot analysis performed using fatostatin and PF429242 at 72 h time point led to significant decrease in the levels of the active form of SREBP1 and its downstream signaling proteins such as FAS, SCD-1 and HMGCoAR and the mutant form of tumor suppressor protein, p53, levels in comparison to the levels observed in vehicle treated control group of MIA PaCa-2 pancreatic cells over the same time period. Our *in vitro* data suggest that SREBP1 may

contribute to pancreatic tumor growth and its inhibitors could be considered as a potential target in the management of pancreatic cancer cell proliferation.

INTRODUCTION

Pancreatic cancer is the 4th leading cause of cancer-related death in the USA, with only a 6% survival rate in 5 years [1]. The low survival rate is due to early recurrence and metastasis of pancreatic carcinoma, and also the late stage diagnosis due to its asymptomatic nature [2]. Over the past few years, several targets such as the epidermal growth factor receptor (EGFR), Phosphatidylinositol 3-kinase (PI3K), protein kinase B (Akt) the mechanistic target of rapamycin (mTOR) and B-Raf have been developed and tried for the inhibition of different cancers. Currently, the EGFR inhibitor, erlotinib, in combination with gemcitabine has been approved for the treatment of pancreatic cancer [3]. While inhibiting these upstream targets has been successful for treating several types of cancers, these therapies are either ineffective or lead to a development of resistance over time. Knowing the complex tumor biology of pancreatic cancer, identification of new targets and therapies is an urgent unmet medical need.

It is now a well-established fact that tumor cells readjust their metabolic pathways to meet the need of cancer cells [4]. In malignant cells, mainly lipid metabolism gets reprogrammed to meet high metabolic demands of cancer cells and the lipogenic phenotype is a significant characteristic of tumors. Tumor cells also divert glucose and glutamate metabolism pathways to serve in lipid biogenesis process [5]. Sterol regulatory element-binding proteins (SREBPs) are key transcription factors that control lipid homeostasis [6]. There are two types of SREBPs, SREBP1, which mainly regulates fatty acid synthesis, and SREBP2, which activates cholesterol

synthesis [7]. The elevated lipid biogenesis regulated by SREBP1 is considered a major characteristic of malignancies including prostate cancer, endometrial cancer, and glioblastoma [8] ; [9]. SREBP1 acts as a central regulator in integrating glucose metabolism regulated by PI3K/Akt signaling pathway and fatty acid synthesis by Myc-regulated glutamine metabolism [10]. The active N-terminal region of SREBP1 localized in the nuclei upregulates expression of several downstream target genes such as fatty acid synthase (FAS) and steaoryl-CoA-desaturase-1 (SCD-1) [11]. The oncogenic receptor tyrosine kinase (RTK)/PI3K/Akt pathway increases the expression of SREBP-1 which in turn upregulates ATP citrate lyase (ACL), Acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) to promote fatty acid synthesis and increase low density lipoprotein receptor expression to increase cholesterol uptake by these malignant cells. The fatty acids and phospholipids promote cell growth and survival. A recent study also showed that inhibition of upstream events such as EGFR, PI3K, and Akt activation lead to drastic reduction in the expression of SREBP1 in cancer cells [11]. While a clear positive correlation between SREBP-1 level and pancreatic cancer cell proliferation has been addressed [6], whether the inhibitors of SREBP1 would affect pancreatic cell has not been explored so far.

Therefore, we analyzed the effect of two known inhibitors of SREBP1, fatostatin and PF429242, on a human pancreatic cancer cell line, MIA PaCa-2 cells. Fatostatin is a non-sterol synthetic diarylthiazole derivative which inhibits SREBP1 maturation and its nuclear translocation [12]. PF429242 is a reversible, competitive aminopyrrolidineamide inhibitor of site-1 protease (S1P), which inhibits endogenous SREBP processing [13]. We investigated the effect of these two

inhibitors on pancreatic MIA PaCa-2 cells' viability as well as their effects on SREBP1 activation and its key downstream targets.

RESULTS

3.1.1 Fatostatin and PF429242 both display concentration and time-dependent further increases in their inhibitory effects in the MIA PaCa-2 cell line

First, we determined the cytotoxic effect of fatostatin and PF429242 in the MIA PaCa-2 cell line as a function of time by incubating them for varying time intervals of 24, 48 and 72 h at concentration ranges (10–320 μM) employing the CCK-8 assay. While significant inhibition ($P < 0.001$) was observed even at 24 h period of incubation using either agent at their lowest concentration (10 μM) employed in the study (Fig. 1A and B upper panel) both agents display concentration and time-dependent further increases in their inhibitory effects (Fig. 1A–F). The IC_{50} values were found to be 14.5 μM and 24.5 μM for fatostatin and PF429242, respectively at 72 h time of incubation.

3.1.2 Western blot analysis showed a significant decrease in active form of SREBP-1 and its downstream signaling proteins FAS and HMGCoAR

Western blot analysis showed a significant decrease in active form of SREBP1 ($P < 0.001$) and its downstream signaling proteins FAS ($P < 0.001$), SCD-1 ($P < 0.001$) and HMGCoAR ($P < 0.001$) in MIA PaCa-2 cells when compared to the levels of these protein seen in normal mice pancreatic tissue homogenates (N). Treatment with both fatostatin (IC_{50} 14.5 μM for 72 h) and PF429242 (IC_{50} 24.5 μM for 72 h) significantly decreased the levels of active form of SREBP-1 ($P < 0.05$ and $P < 0.01$, respectively;Fig. 2B), FAS ($P < 0.05$ and $P < 0.05$, respectively; Fig. 2C), SCD-1

($P < 0.01$ and $P < 0.001$, respectively; Fig. 2D), HMGCoAR ($P < 0.01$ for PF429242; Fig. 2E) and p53 ($P < 0.05$ and $P < 0.001$, respectively; Fig. 2F) in MIA PaCa-2 cells in comparison to vehicle treated control group of MIA PaCa-2 cells maintained over the same time period.

3.2.1 FIGURE 1

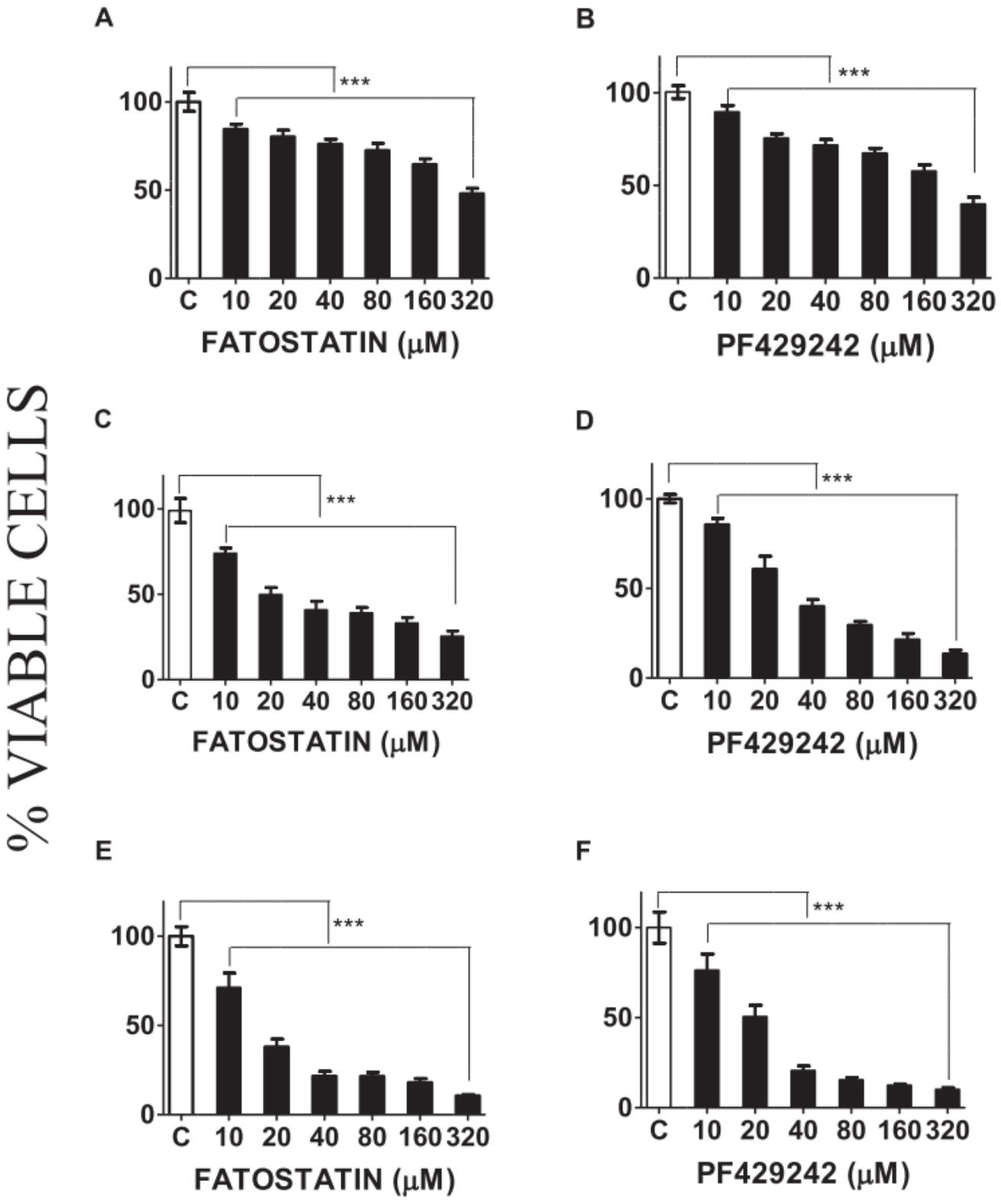

FIGURE LEGEND 1

Effects of inhibition of MIA PaCa-2 pancreatic cancer cell proliferation *in vitro* attained with increasing concentrations of either fatostatin (10–320 μM) or PF429242 (10–320 μM) for 24 h (upper panel Fig. 1A and Fig. 1B), 48 h (middle panel Fig. 1C and Fig. 1D) and 72 h (lower panel Fig. 1E and Fig. 1F) periods of incubation. Each bar diagram represents mean ± standard deviation (S.D.) of mean values of % of viable cells (n = 3 separate experiments performed in triplicate). ***$P < 0.001$ compared to respective vehicle-treated control group (C) of MIA PaCa-2 pancreatic cells.

3.2.2 FIGURE 2

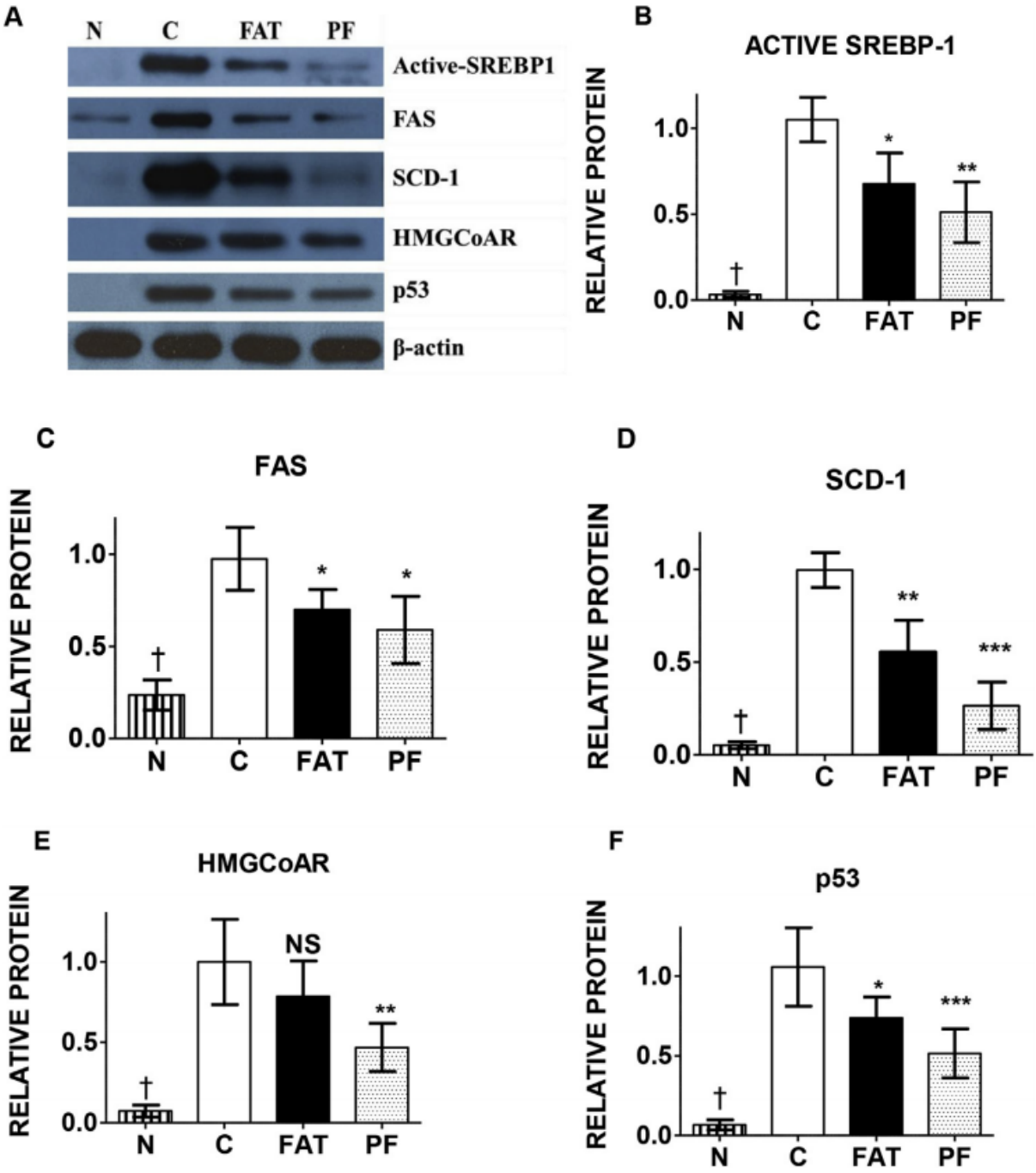

FIGURE LEGEND 2

Western blot data for the effects of fatostatin and PF429242 on MIA PaCa-2 pancreatic cancer cell line. The representative Western blots for the levels of active SREBP-1, fatty acid synthase (FAS), stearoyl-CoA-reductase-1 (SCD-1), HMGCoAR, tumor suppressor protein, p53, along with b-actin determined in normal mouse pancreatic tissue homogenate (N), in vehicle-treated control group (C), fatostatin (FAT IC50 - 42.5 mM for 48 h) and PF429242 (PF IC50 47 mM for 48 h) treated MIA PaCa-2 pancreatic cells are shown (Fig. 2A). The Western blot data analyzed from several experiments are presented as bar diagrams [mean ± S.D. values, n = 3) for the levels of active SREBP-1 (Fig. 2B), FAS (Fig. 2C), SCD-1 (Fig. 2D), HMGCoAR (Fig. 2E), p53 (Fig. 2F) determined seen in normal mouse pancreatic tissue homogenate (N) vs. the control group of vehicle treated group (C) or fatostatin (FAT) or PF429242 (PF) treated MIA PaCa-2 pancreatic cells maintained in vitro in culture. T¸ denotes P < 0.001 in normal mouse pancreatic tissue homogenate (N) compared to the levels noted in vehicle treated control group MIA PaCa-2 pancreatic cancer cells (C). *P < 0.05, **P < 0.01, ***P < 0.001 in FAT or PF treated cells compared to respective vehicletreated control group (C) of MIA PaCa-2 cells.

MATERIALS AND METHODS

3.3 materials

MIA PaCa-2 cell line was obtained from Sigma-Aldrich (Sigma-85062806). Dulbecco's Modified Eagle Medium (Hyclone DMEM), fetal bovine serum (FBS) were purchased from HyClone (Cat No: SH30024.01). Cell Counting-8 (CCK-8) kit was purchased from Dojindo (Cat No: LT807). Fatostatin was obtained from TOCRIS BIOSCIENCE (Catalog No:4444). PF429242 was obtained from SIGMA (Catalog No: SML0667).

3.3.1 **Maintenance of tumor cell line**

MIA PaCa-2 cell line was authenticated by Sigma-Aldrich, Canada (Invoice No. 536284272; dated 06/22/2015). These cells were grown in Hyclone DMEM with 10% FBS and cultured in a humidified atmosphere of 5% CO_2 at 37 °C.

3.3.2 **Cytotoxicity assay**

MIA PaCa-2 cells (4000 cells/well) were seeded in 96-well plates with the medium mentioned above. After overnight attachment, the cells were treated with the medium containing either fatostatin (0–320 μM) or PF429242 (0–320 μM) for 24, 48 and 72 h (6 wells/concentration). Fatostatin (1 mM) was initially dissolved in dimethyl sulfoxide while PF429242 was dissolved in sterile water and further dilutions of each inhibitors were made in Dulbecco's modified essential medium. The viable cell number was measured using the CCK-8 assay kit. Absorbance was measured at 450 nm using a microplate reader (Spectral Max M5). The optical density of each well was measured to represent the proliferation of the cells. All the experiments were performed in triplicate.

3.3.3 Western blot analysis

MIA PaCa-2 cells were plated in 10 cm Petri dishes. After overnight attachment, the cells were treated with either vehicle (control), PF429242 or fatostatin for 72 h. The concentrations of fatostatin and PF429242 employed were chosen based on the IC50 values derived from preliminary studies. For total protein extraction, cells were lysed in RIPA buffer (Cell Signalling, #9806, 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, 1 μg/ml leupeptin) supplemented with protease inhibitor cocktail (Cell Signaling,

#5871). Normal pancreatic tissues were obtained from 2 months old male and female C57BL/6 mice. The mice were euthanized by cervical dislocation under mild anesthesia (isoflurane), the pancreas was excised out and processed for whole cell lysates similar to the Mia PaCa-2 cell lysates as mentioned above. Total protein extracts were quantified using the Bradford assay with bovine serum albumin as standard. Western blot experiments were carried out using the Mini Trans-Blot cell BIO-RAD. The following primary antibodies were used: anti-FAS (Santa Cruz, sc-48357; dilution 1:500), anti-SCD1 (Abcam, ab19862; dilution 1:1000), anti- HMGCoAR (Abcam, ab174830; dilution 1:1000), anti-SREBP1 (Santa Cruz, sc-365513, 1; 500) and anti-p53 (Abcam, ab1101; dilution 1:1000). Anti-beta Actin (Abcam,ab8227; dilution 1:1000) was used for normalization. All the experiments were performed in triplicate.

3.3.4 Statistical analysis

All the statistical analyses were conducted using Graphpad Prism 6 software (GraphPad Software, San Diego, CA). Data are presented as mean ± standard deviation (SD). For western blots, the protein expressions for various conditions were compared by one-way ANOVA test. *P* values < 0.05 were considered as statistically significant.

DISCUSSION

There is increasing evidence that cancer cells engage in increased lipogenesis to become independent of systemic regulation, leading to rapid cell proliferation and enhanced tumor growth [7] ; [10]. Therefore, recent studies have explored the role of SREBP1 as a novel molecular target in several cancers. The data from the present study shows that SREBP1 and its downstream signaling protein were increased in pancreatic cancer cells and the inhibition of SREBP1 overactivation using either fatostatin or PF429242 led to a significant reduction in the downstream signaling proteins with both agents displaying a similar degree of time and concentration-dependent inhibition of MIA PaCa-2 pancreatic cell proliferation.

Precursor SREBP1 is bound to the ER membranes, which upon activation undergoes two-step cleavage and releases the N-terminal active form of SREBP1 in the nucleus. Fatostatin decreases the amounts of fatty acid, triglyceride and low-density lipoprotein by disturbing the activation of SREBP1 [12]. PF429242 a reversible, competitive inhibitor of endogenous processing of SREBP1 decreases lipid biosynthesis [13].

SREBP genes also regulate expression of HMGCoAR, a rate-limiting enzyme of the mevalonate pathway [14]. HMGCoAR is a common target of statins used to treat high levels of cholesterol. Statins have been shown to exert anticancer activity *in vitro* and in preclinical models [15]. However, clinically, it was confirmed that statins did not have any impact on reducing cancer burden [16] ; [17]. An increase in SREBP1 induces the expression of several enzymes involved in lipid synthesis such as FAS, HMGCoAR, and SCD-1. We observed that inhibition of SREBP1 led to decreased expression of these enzymes in the pancreatic cancer cell line.

p53, a tumor suppressor oncogene, is mutated in more than 75% of cancer cases [18]. The presence of a mutant form of p53 is always detected in rapidly growing pancreatic cancer cases [19]. The Hippo signaling and p53 pathways influence the expression of SREBP mRNA and SREBP transcriptional activity [14]. Wild-Type p53 represses transcription of SREBP1 [20]. p53 deletion has been shown to increase the expression of SREBP1 and its downstream targets (including FAS) in mice [20]. In the case of mutant p53, gain-of-function mutants $p53^{R273H}$ and $p53^{R280K}$ bind with SREBPs and activate their transcriptional activity. As shown in Fig. 2, MIA PaCa-2 cells show high expression of p53 and perhaps mutated p53 [12]. Both fatostatin and PF429242 significantly reduced the expression of p53. We also observed two sterol regulatory element sequences (5′-ATCACCCCAC-3′) with 100% homogeneity in p53 gene sequence at 1904–1911 and 2350–2358 base pairs in BLAST analysis suggesting a potential regulatory role of SREBP1 over p53 gene expression. Thus, we speculate that since the majority of p53 is mutated in tumor cells, the reduction in the level with SREBP1 inhibitors most likely reflects a reduction in the mutant form of p53. Li et al. have also noted that fatostatin has antitumor effects in mutant p53 metastatic prostate cancer cells [12]. It is noteworthy that in our present study we have supportive data that the normal pancreatic tissues obtained from mice expressed a very low level of SREBP1 as well as the downstream targets FAS, HMGCoAR and SCD-1. This clearly shows that SREBP1 and its downstream targets are overexpressed and activated in cancer cells as a result of the changes in the lipid metabolism pathways described in the earlier sections.

In summary, the data from the present study using two selective inhibitors of SREBP1 confirm that both agents exert selective cytotoxicity in MIA PaCa-2 pancreatic cancer cells along reduction of proteins involved in lipid biosynthesis. These data suggest SREBP1 inhibition could

serve as a potential target to explore in combination with the first line chemotherapy to improve prognosis in pancreatic cancers.

Future Directions

GBM is one of the deadliest malignancies, with a very short life expectancy after it is diagnosed. Unfortunately, there is no effective treatment for GBM as average survival after surgery, chemotherapy, and radiotherapy is estimated to be about 14-15 months. Recent extensive media coverage on GBM, following diagnosis of celebrities and politicians such as famous singer Gordon Downie and Senator John McCain with malignant brain tumors has drawn a great deal of attention to this disease and accelerated efforts in drug discovery. To pursue my current research, I propose and envision the following potential research avenues and goals:

1. To study effects of SREBP1 inhibitors on patient GBM tumor stem cells. One of the major reasons for conventional chemotherapy failure in cancers, including GBM tumors, is the propagation and proliferation of unresponsive tumor stem cells. We are planning to gain access to small sections of GBM tumors resected from patients and expose isolated stem cells to various SREBP1 inhibitors alone and in combination with each other or with conventional chemotherapeutic agents such as TMZ.
2. Using immune system compromised xenograft mice is considered the next transitional step from *in vitro* study to *in vivo* model. I will screen and evaluate the effectiveness of SREBP1 inhibitors (alone or in combination) in a NoD-SCID xenograft mouse model.
3. As an ongoing SHRF CID collaboration between my supervisor and Dr. Maruti Uppalapati (Department of Pathology, University of Saskatchewan), they are investigating short

peptide domains as novel SREBP1 inhibitors. I can screen any peptide fragments generated from this collaboration against GBM using in vitro and in vivo models.

4. Although Indip peptide crosses the BBB because of TAT fusion, using Indip and other SREBP1 inhibitors as well as TMZ loaded nanoparticles to improve drug delivery into the CNS can also be considered a feasible study for the continuation of this project.

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